Genetic Analysis of Alcohol-Metabolizing Enzymes in Thermophilic Bacteria and Acetic Acid Bacteria

A Doctoral Thesis

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Abbreviation List

ADH	Alcohol Dehydrogenase
ADH-T	Thermostable Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
ALDH-T	Thermostable Aldehyde Dehydrogenase
Arg	Arginine
ATP	Adenosine Triphosphate
A. pasteurianus	*Acetobacter pasteurianus*
B. subtilis	*Bacillus subtilis*
CCCP	Carbonylcyanide-m-Chlorophenylhydrazone
Cys	Cysteine
DNA	Deoxyribonucleic Acid
DEAE	Diethylaminoethyl
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
G. europaeus	*Gluconacetobacter europaeus*
G. stearothermophilus	*Geobacillus stearothermophilus*
Glu	Glutamic Acid
His	Histidine
IPTG	Isopropyl β-D-Thiogalactopyranoside
L broth	Luria-Bertani broth
Lys	Lysine
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NTG	*N*-Methyl-*N*′-Nitro-*N*-Nitrosoguanidine
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulfate
Ser	Serine
T. kodakaraensis	*Thermococcus kodakaraensis*
Tc	Tetracycline
Thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan

GENERAL INTRODUCTION

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are ubiquitous enzymes that are widely distributed among several genera. Horse liver ADH has been much researched, and its catalytic mechanism is well known. X-ray crystallography study of horse liver ADH has revealed that the residues within its catalytic domain provide ligands to the catalytic zinc atom; Cys46, His67, and Cys174. The catalytic zinc atom is at the bottom of this active pocket with zinc-bound water molecule. This water molecule is hydrogen-bonded to the side chain of Ser48, which in turn is hydrogen-bonded to His51 (Fig. 1). The deprotonation of the catalytic zinc-water is facilitated by His51 residue acting through a hydrogen-bonded network to relay the proton to solvent (1, 2). The second zinc atom of the subunit is liganded by four sulfur atoms from Cys97, Cys100, Cys103, and Cys111.

Aldehydes are highly reactive and generated from multitudes of endogenous and exogenous sources. They have a variety of effects on the biological systems. Although some aldehydes such as retinaldehyde have beneficial effects, several aldehydes have deleterious effects such as cytotoxicity, mutagenicity, and carcinogenicity. Diverse enzymes have evolved to metabolize aldehydes to their less reactive forms. Among the most effective pathways for aldehyde metabolism is their oxidation to carboxylic acids by ALDHs (3). Crystallographic analyses have revealed the catalytic mechanism of ALDH. The overall mechanism of the reaction catalyzed by the hydrolytic ALDHs involves three main steps: (i) nucleophilic attack of the thiol group of the catalytic cysteine on the carbonyl carbon of the aldehyde substrate; (ii) hydride transfer from the tetrahedral thiohemiacetal intermediate to the pyridine ring of NAD(P)+; and (iii) hydrolysis of the resulting thioester intermediate (deacylation) (4).

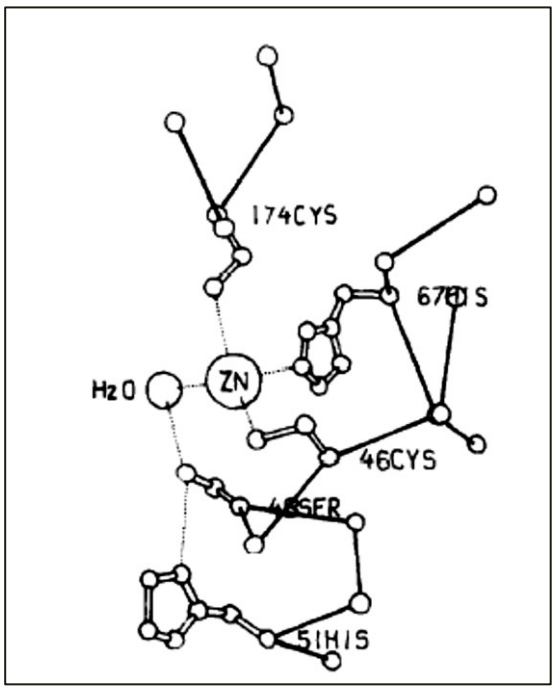

FIG. 1. Stereo diagram showing the spatial orientation of the zinc-bound water molecule, the side chains of Ser48 and His51, and the hydrogen bond system between these groups, predicted by Eklund et al (1).

Acetic acid bacteria are gram-negative bacteria belonging to the group of *Proteobacteria*. They are capable of oxidizing ethanol and sugars to corresponding organic acids under aerobic condition (5). The kinetic study suggested that ADH and ALDH played a major role in alcohol metabolism of acetic acid bacteria (6, 7). They contained two systems for conversion of ethanol via aldehyde to acetic acid. One system was linked to the cytoplasmic membrane; the other system was in solution of the cytoplasm (8). They have an efflux pump for acetic acid at the cytoplasmic membrane, which is specific for acetic acid and is driven by a proton motive force independently of ATP; it is responsible for acetic acid resistance (9).

Two acetic acid bacteria, *Acetobacter pasteurianus* and *Gluconacetobacter europaeus*, are generally used for industrial vinegar production because they can efficiently oxidize ethanol to acetic acid and show strong resistance to ethanol and acetic acid. *A. pasteurianus* has been used in the traditional fermentation. *G. europaeus* has been used for the production of high-acidity vinegar in submerged bioreactors because it has extremely

strong ethanol-oxidizing ability and ethanol/acetic acid resistance as compared with other species. Therefore, *G. europaeus* is a main bacterium for industrial vinegar production in the world. However, studies on molecular genetics and biochemical analysis of *G. europaeus* have been limited.

A targeted gene disruption system, using uracil auxotroph as a result of pyrimidine biosynthesis pathway genes impairment, was shown to be available for several microbes (10). This approach does not require any exogenous drug resistance genes. Orotate-phosphoribosyltransferase (OPRTase; encoded by *pyrE*) and orotidine-5′-mono-phosphate decarboxylase (OMPdecase; encoded by *pyrF*) catalyze the last two steps of pyrimidine biosynthesis. A bacterial orotic acid analogue, 5-fluoroorotic acid (5-FOA) is converted to 5-fluorouridine monophosphate (5-FUMP), which exhibits a bactericidal effect, by these enzymes. Mutants with defects in either of these enzymes are resistant to 5-FOA, but they are uracil auxotroph. Recently, a selection system utilizing these properties was successfully established in *G. europaeus* (11).

Molecular biology studies have shown that microorganisms can be transformed with artificial plasmids containing genes necessary for a specific synthetic production. However, the major problem with these plasmids is their instability in transmitting their properties to their daughter cells in a controlled manner during the cell division of the microorganisms. As a result, a large number of daughter cells, which does not contain the plasmid of interest, are produced during the fermentation. On a laboratory scale, this loss in desired plasmid can be countered by supplementing the culture medium with an antibiotic reagent corresponding to the antibiotic resistant gene of the plasmid. However, a large-scale addition of antibiotics in fermentations is disadvantageous. Therefore, addressing recombinant plasmid stability without adding antibiotic reagents is the most critical issue for a fermentation process.

In Chapter 1, the rational shift of the optimum pH was performed by substituting the catalytic amino acids of ADH from *Geobacillus stearothermophilus*.

In Chapter 2, the thermostable ALDH gene of *G. stearothermophilus* was cloned and substrate specificity was investigated.

In Chapter 3, a new method for stabilizing recombinant plasmid was established and applied for tryptophan production in *Escherichia coli*, and a thought-provoking approach for *G. europaeus* has been discussed.

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CHAPTER 1

Catalytic Metabolism of the NAD-Dependent Alcohol Dehydrogenase and Rational Shift of the Optimum pH

INTRODUCTION

Various thermostable alcohol dehydrogenases (ADH-Ts) have been analyzed for the industrial production of alcohol (1-3), including chiral alcohol (4). *Geobacillus stearothermophilus* NCA1503 was found to produce an ADH-T amounting to 1 to 2% of soluble cell protein. This strain produced ethanol from sucrose or glucose as a carbon source under anaerobic conditions at high temperatures (1, 5). Two types of ADH have been isolated from *G. stearothermophilus* NCA1503 and DSM2334 (6). ADH-T from NCA1503 showed enzymatic, structural, and immunological properties different from those of the ADH from DSM2334. The ADH from DSM2334 is active with primary alcohols, including methanol (6, 7). The gene for ADH from DSM2334 has been cloned in *Escherichia coli* (8). In this work, the ADH-T gene (*adhT*) from *G. stearothermophilus* NCA1503 was cloned in *Bacillus subtilis*.

The ADH reaction mechanism was originally studied with horse liver ADH by X-ray crystallographic analysis and a kinetic study. The catalytic domain of horse liver ADH is composed of the catalytic zinc-water, the side chain of Ser48, and the imidazole ring of His51. The deprotonation of the catalytic zinc-water is facilitated by the imidazole ring of His51 acting through a hydrogen-bonded network to relay the proton to solvent (7, 9, 10). By comparing amino acid sequences of ADH-T with horse liver ADH and other ADHs, the catalytic system of ADH-T was inferred.

Here, we describe the molecular cloning and nucleotide sequencing of the ADH-T gene (*adhT*) from *G. stearothermophilus* NCA1503, a comparison of the deduced amino

acid sequence with the sequences of other ADHs, prediction of the catalytic system of ADH-T based on the crystallographically determined model of the horse liver ADH, confirmation of the putative catalytic system by replacing the catalytic amino acids of ADH-T by using site-directed mutagenesis, and construction of a modified enzyme that exhibits a pH profile different from that of the wild-type ADH-T.

MATERIALS AND METHODS

Bacterial strains and plasmids.

G. stearothermophilus NCA1503 (11) was used as a DNA donor. *B. subtilis* MI113 (*arg-15 trpC2 hsrM hsmM*) (11) and M1112 (*leuA8 thr-5 arg-15 recE4 hsrM hsmM*) (12) were used as host cells in gene cloning. Since *B. subtilis* MI112 is deficient in DNA recombination, it was used as the host cell to stably carry the recombinant plasmid. A low-copy-number plasmid, pTB524 (Tc^r; coding for tetracycline resistance) (13), which has a *Bam*HI site suitable for gene cloning, was used to construct the gene bank of *G. stearothermophilus* NCA1503. pTB522 (Tc^r) (14), which has a *Hin*dIII site for cloning, and pTB524 were used for subcloning of the gene. *E. coli* TG1 (*supEA* (*lac-proAB*) *hsdΔ5 F′ traD36 proAB*$^+$ *lacIq lacZΔM15*) (15) and M13 mpl8 and M13 mp19 (15) were used as a host cell and phages to subclone the gene for nucleotide sequencing.

Media.

G. stearothermophilus NCA1503 was grown at 55°C in modified L broth containing tryptone (20 g/liter), yeast extract (10 g/liter), and NaCl (5 g/liter), and the pH was adjusted to 7.3 with 2 N NaOH. *B. subtilis* M1113 and M1112 were grown in L broth (11) at 37°C. Solid medium contained 20 g of agar per liter for growth at 55°C and 15 g of agar per liter for growth at 37°C.

Transformants of *B. subtilis* with pTB524, pTB522, or their derivatives carrying the tetracycline (Tc) resistance gene were grown in L broth containing Tc (25 μg/ml).

Detection of ADH-producing colonies on plates.

ADH-producing colonies were selected on modified aldehyde indicator plates as described by Conway et al. (16), with slight modification. The plates were composed of antibiotic medium 3 (17.5 g/liter) (Difco Laboratories, Detroit, MI, USA) acting as a buffer (pH 7.0), ethanol (20 ml/liter), pararosaniline (50 mg/liter) (Sigma Chemical Co., St. Louis, MO, USA), and sodium hydrogen sulfite (250 mg/liter). Ethanol diffuses into cells and can be converted by ADH to acetaldehyde, which reacts with the reagent to form Schiff base intense red.

Preparation of plasmids and chromosomal DNA.

Either the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation was used to prepare plasmid DNA, whereas chromosomal DNA was prepared as described elsewhere (17, 18).

Transformation of *B. subtilis*.

For transformation of *B. subtilis*, competent cells were prepared as described previously (18). Tc^r transformants were transferred on the modified aldehyde indicator plates and incubated at 37°C for 5 hours to check colony color turning intense red.

Nucleotide sequencing.

DNA was sequenced by the dideoxy method of Sanger et al. (19) with the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, OH, USA). After digestion

with restriction enzymes, DNA fragments were subcloned into M13 mpl8 or M13 mpl9. *E. coli* TG1 was used as a host cell.

Site-directed mutagenesis.

Point mutations were introduced into a gene with an oligonucleotide-directed in vitro mutagenesis system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Active staining of ADH.

Active staining of ADH was performed according to the method described by Dowds et al. (8). Crude enzymes were run on a 6% polyacrylamide gel with solutions and reagents from which sodium dodecyl sulfate (SDS) was omitted. The gel was stained for ADH activity by an alcohol-dependent nitroblue-tetrazolium procedure. The gel was soaked in 500 mM Tris-HCl (pH 8.8) at 4°C for 15 min and then incubated at 37°C for 30 min in a staining solution containing 150 mM Tris-HCl (pH 8.8), NAD (0.132 mg/ml), nitroblue-tetrazolium (0.163 mg/ml), phenazine methosulfate (0.03 mg/ml), and ethanol (10 ml/liter). These reagents were purchased from Sigma Chemical Co.

Enzyme purification.

ADH-T and its derivatives were purified from the transformants of *B. subtilis* MI112. They were grown to the stationary phase at 37°C in L broth containing Tc (25 μg/ml), harvested by centrifugation (10,000×g, 10 min) at 4°C, and washed in 20 mM potassium phosphate buffer (pH 7.8). The cell pellet was suspended in the phosphate buffer containing lysozyme (l mg/ml) and DNase I (10 U/ml) and incubated at 37°C for 30 min. After centrifugation (55,000×g, 30 min), the supernatant was heated at 60°C for 10 min and again

centrifuged (20,000×g, 10 min) at 4°C. The crude enzyme was purified by DEAE-cellulose (DE52, Whatman BioSystems Ltd., Maidstone, Kent, UK) ion-exchange column chromatography, equilibrated with 20 mM potassium phosphate buffer (pH 7.8). The enzyme was eluted with a linear gradient (0 - 1 M) of potassium chloride dissolved in the phosphate buffer. Active fractions were dialyzed overnight at 4°C in 20 mM potassium phosphate buffer (pH 7.8). The final enzyme preparation was confirmed to be homogeneous by SDS polyacrylamide gel electrophoresis (PAGE).

According to the method described above, wild-type ADH-T and mutant enzymes Thr40Ser and His43Arg were purified to homogeneity.

Assay of ADH activity.

ADH activity was assayed by monitoring alcohol-dependent NAD reduction or acetaldehyde- or ketone-dependent NADH oxidation at 340 nm (6, 20, 21). ADH-activity (U) was expressed as μmol of NADH produced or decreased per min, using a molar absorption coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$. The assay was performed at 55°C in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 1 mM NAD, and 100 mM each alcohol. The aldehyde or ketone reductase (reverse reaction of alcohol dehydrogenase) assay was performed at 55°C in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.2 mM NADH, and 100 mM acetaldehyde or each ketone (20). Concentrations of substrates and coenzymes were confirmed to be excessive by measuring enzyme activity under various concentrations of substrates and coenzymes. The standard ADH assay was performed at 55°C in a reaction mixture which contained 100 mM potassium phosphate buffer (pH 7.8), 1 mM NAD, and 100 mM ethanol. To examine the pH profile of the enzyme, 100 mM potassium phosphate buffer or 100 mM glycine-KOH buffer having various pH was used.

Protein assay.

The protein concentration was measured by the method of Lowry et al. (22) with bovine serum albumin as the standard.

Other procedures.

Procedures for digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis, and SDS-PAGE were described elsewhere (17, 23-25). Unless otherwise specified, all chemicals used in this work came from sources described in a previous paper (26).

Nucleotide sequence accession number.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D90421.

RESULTS

Cloning of the ADH gene from *G. stearothermophilus* NCA1503.

Chromosomal DNA of *G. stearothermophilus* was partially digested with *Sau*3AI, and fragments of approximately 6 kb were isolated and purified by Gene-Clean (Bio101 Inc., La Jolla, CA, USA). These fragments were ligated into the *Bam*HI site of pTB524. The ligation mixture was used to transform *B. subtilis* MI113. Of 3,000 Tc^r transformants of *B. subtilis*, one ADH-positive clone was found on the modified aldehyde indicator plates (Fig.1). The transformant carried a recombinant plasmid containing an insert of about 7 kb. A lysate of the candidate cell, subjected to electrophoresis, showed a band of ADH-active staining at the same position as that of a DNA donor strain, *G. stearothermophilus* NCA1503 (photograph not shown). The level of ADH-activity of the recombinant plasmid carrier (1.48 U/mg of dry cell) was about nine-fold higher than that of the DNA donor (0.17 U/mg of dry cell), whereas

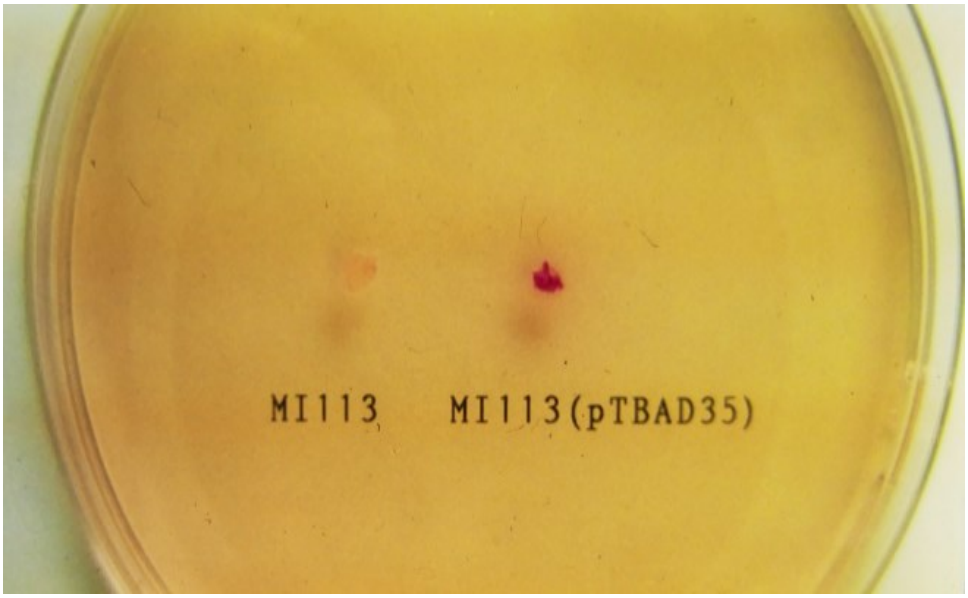

FIG. 1. Detection of ADH-producing colonies on the aldehyde indicator plate.
Ethanol diffusing into cell can be converted by ADH to acetaldehyde, which reacts with the reagent (pararosaniline) to form Schiff base intense red. Host cell (*B. subtilis* MI113) showed a slightly pink. Transformants harboring a recombinant plasmid (pTBAD35) showed intense red.

the host cell showed little activity (less than 0.002 U/mg of dry cell). The candidate produced a MW 35,000 protein, which could also be found in the DNA donor but not in the host cell, and moreover, the protein was thermostable (Fig. 2). The recombinant plasmid was designated pTBAD70. It was concluded that pTBAD70 carried the ADH-T gene (*adhT*) from *G. stearothermophilus* NCA1503.

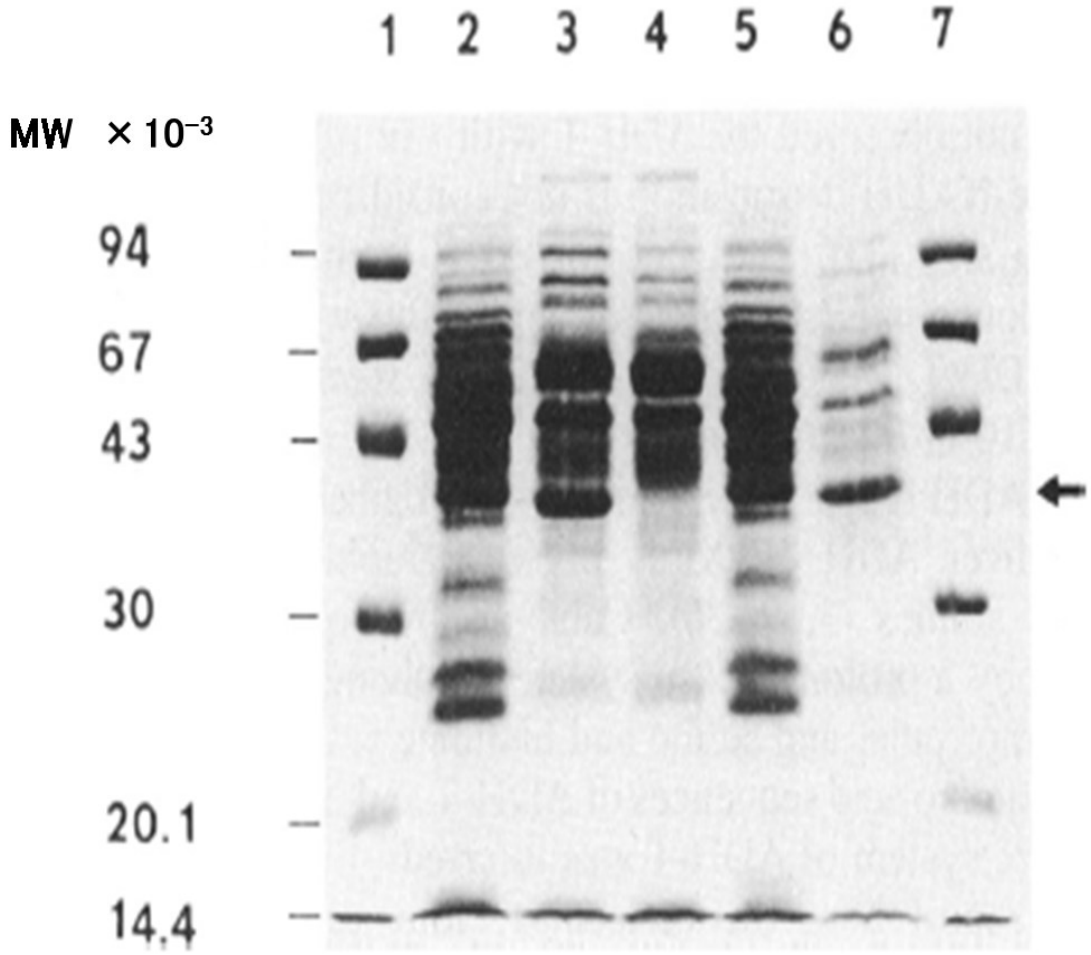

FIG. 2. SDS-PAGE analysis of cell extracts.

Each lane contains 5 µl of cell extract. Lanes: 1 and 7, molecular weight markers; 2, *G. stearothermophilus* NCA1503; 3, ADH-positive transformant of *B. subtilis* M1113; 4, B. subtilis M1113; 5, cell extract of *G. stearothermophilus* NCA1503, heated (60°C, 10 min) and centrifuged (20,000 x g); 6, cell extract of B. subtilis transformant, heated (60°C, 10 min) and centrifuged (20,000 x g). The arrow indicates the position of ADH-T.

Subcloning of the *adhT* gene.

To analyze the location of the *adhT* gene, I constructed three deletion plasmids from pTBAD70. A *Bam*HI fragment (about 3.5 kb) and a *Hin*dⅢ fragment (about 4.0 kb) from pTBAD70 were subcloned in pTB524 and pTB522, and their recombinant plasmids were designated pTBAD35 and pTBAD40, respectively. pTBAD70-, pTBAD40-, and pTBAD35-harboring cells showed ADH-activity on the aldehyde indicator plate (Fig. 1). In contrast, the strain carrying pTBAD35 *Δ Sph*I, which lacked an *Sph*I fragment (about 0.6 kb) from pTBAD35, showed no ADH-activity. Therefore, the *adhT* gene was considered to be located in the 2.2-kb *Hin*dIII-*Bam*HI fragment including the *Sph*I fragment.

Nucleotide sequence of the *adhT* gene.

The nucleotide sequence of the 2.2 kb *Hin*dIII-*Bam*HI fragment was determined. A large open reading frame was found in the 1.7-kb *Eco*RI-*Bam*HI fragment (Fig. 3). It was composed of 1,011 bp corresponding to 337 amino acids. The molecular weight was estimated to be 36,098, which agreed with the result of SDS-PAGE (Fig. 2). The N-terminal amino acid sequence has been reported elsewhere (27, 28), and the first 40 amino acids were identical to the N-terminal sequence deduced from the nucleotide sequence. The amino acid composition which had been reported previously (6) was also in agreement with the sequencing result in this work. It was therefore concluded that the open reading frame encoded the ADH-T gene (*adhT*). A Shine-Dalgarno sequence was found 10 bases upstream from the translation start site (ATG). Since a large amount of ADH-T was produced, a strong promoter was expected. However, a typical promoter sequence was not found. The sequence resembling typical prokaryotic terminators was found downstream from the open reading frame. The highly AT-rich sequence (about 200 bp) was found at the 5'-flanking region of the open reading frame (Fig. 3).

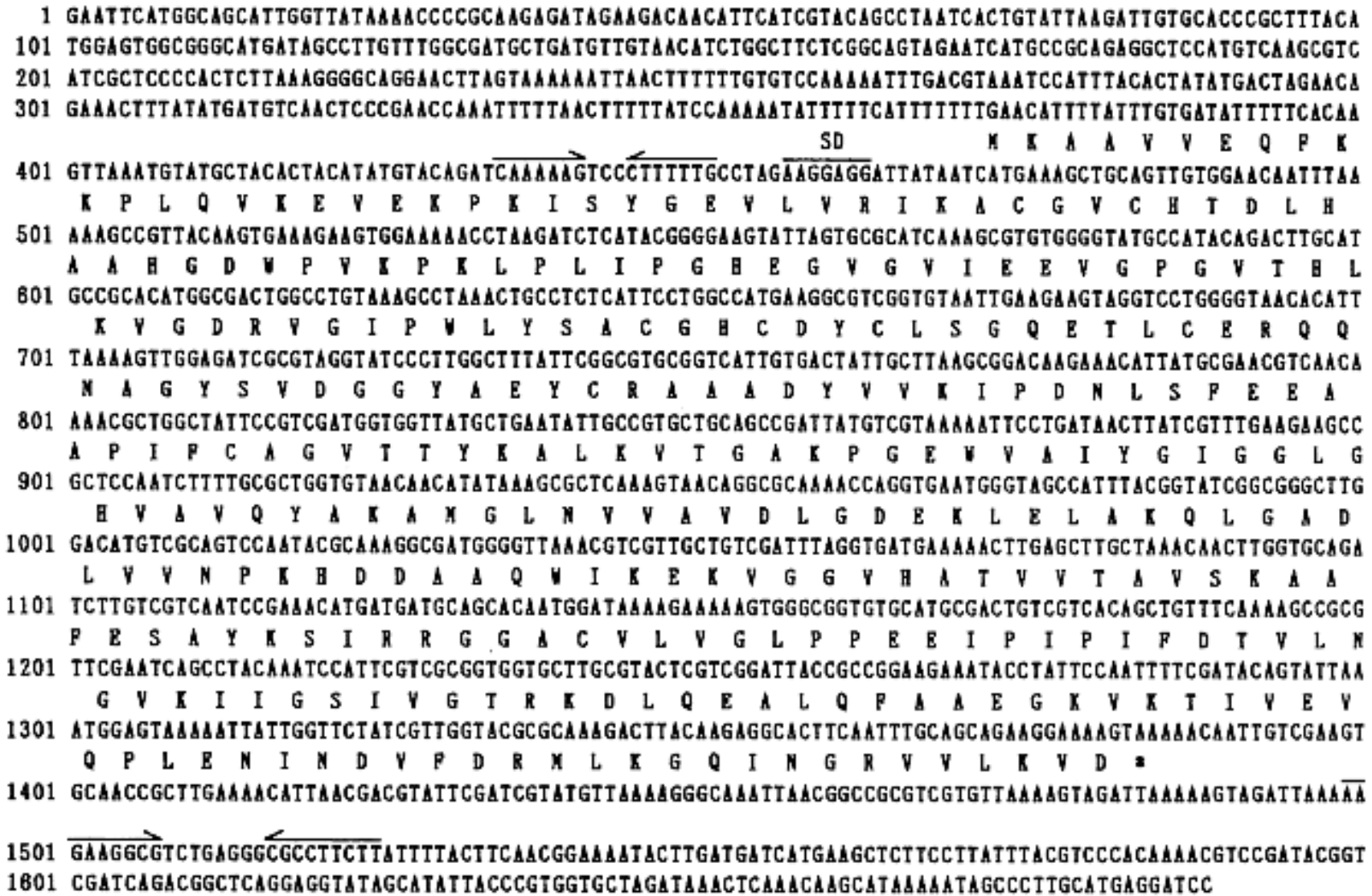
SD

FIG. 3. Nucleotide sequence of the *adhT* gene and the deduced amino acid sequence of the encoded protein.

The amino acid sequence is shown above the nucleotide sequence. A probable Shine-Dalgarno (SD) sequence is indicated by a solid line. The terminator and inverted repeat at the 5'-flanking region are shown by arrows. An asterisk indicates a stop codon.

←

Comparison of the deduced amino acid sequence with the sequences of different ADHs.

Comparison of the primary structures of enzymes with the same function but different origins is useful to determine the amino acids essential for activity, because the active site and substrate binding site are highly conserved (29). ADHs are widely distributed in different organisms and tissues. I compared the amino acid sequences of different ADHs. Most of them showed homology. The deduced amino acid sequence of ADH-T from *G. stearothermophilus* was homologous (55% identity) with that of *Gluconacetobacter europaeus* (32), 56% with *Acetobacter pasteurianus* (33), 32% with *Saccharomyces cerevisiae* (34), 31% with human (35), 34% with horse (36), 27% with *Thermococcus kodakaraensis* (37).

The amino acids indispensable for the catalytic activity of horse liver ADH (7, 36) are highly conserved in the seven ADHs (Fig. 4). The catalytic zinc atom of horse liver ADH is bound by three protein ligands, one sulfur atom each from Cys38 (cysteine at position 38 of ADH-T) and Cys148 and one nitrogen atom from His61. These amino acids are completely conserved. The ligands of the second zinc atom, Cys92, Cys95, Cys98, and Cys106, are also conserved. Furthermore, one of the amino acids participating in the proton release system, a serine residue of horse liver ADH corresponding to position 40 of *G. stearothermophilus* ADH-T, is substituted with threonine. Serine and threonine could play the same function through their hydroxyl group. Another amino acid in the proton release system, His43 of ADH-T, is highly conserved (Fig. 4).

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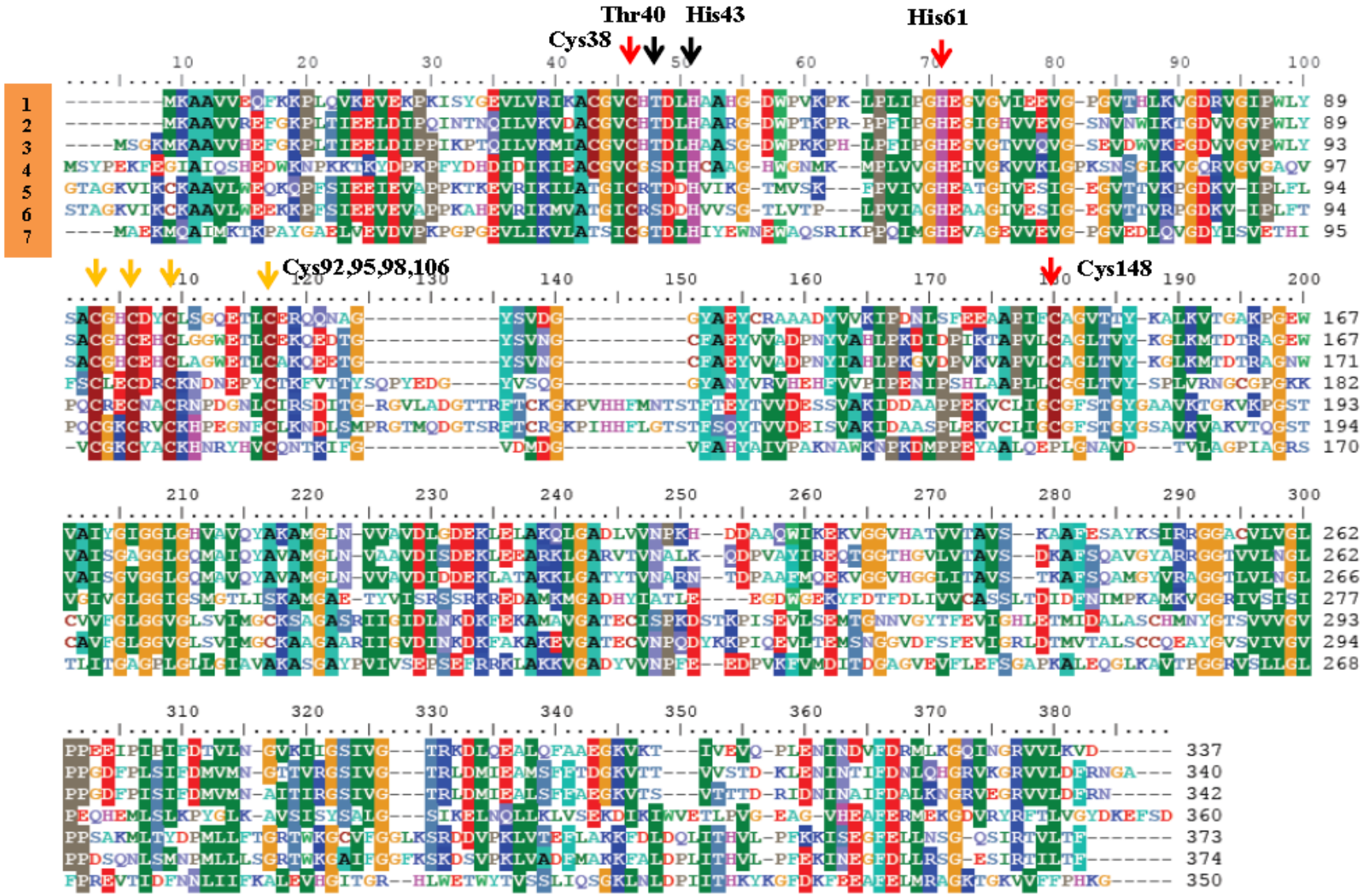
FIG. 4. Comparison of amino acid sequences of seven different ADHs

Lanes: 1, *G. stearothermophilus*; 2, *G. europaeus*; 3, *A. pasteurianus*; 4, *S. cerevisiae*; 5, human; 6, horse; 7, *T. kodakaraensis*. The catalytic amino acids are indicated by arrows.


Thr40
His43
Cys38
His61
Cys92,95,98,106
Cys148
1
2
3
4
5
6
7

A putative reaction mechanism for ADH-T.

According to the argument mentioned above, it is believed that these ADHs, including ADH-T, have the same reaction mechanism as that shown for horse liver ADH (7). The basic reaction mechanism of horse liver ADH is as follows. One equivalent of proton is released per equivalent of ethanol that is oxidized. This proton release is associated with NAD binding and is dissociated from the water molecule bound to catalytic zinc. This proton release from the water molecule occurs via the hydrogen bond system through the side chain (hydroxyl group) of Thr40 to the imidazole ring of His43 (Fig. 5B). Then alcohol binds to zinc as the alcoholate ion, displacing the hydroxyl ion. The zinc atom polarizes the alcoholate so that direct hydrogen transfer and subsequent rearrangement to aldehyde can occur (7, 9). A putative proton release system for ADH-T was predicted by following the mechanism of horse liver ADH.

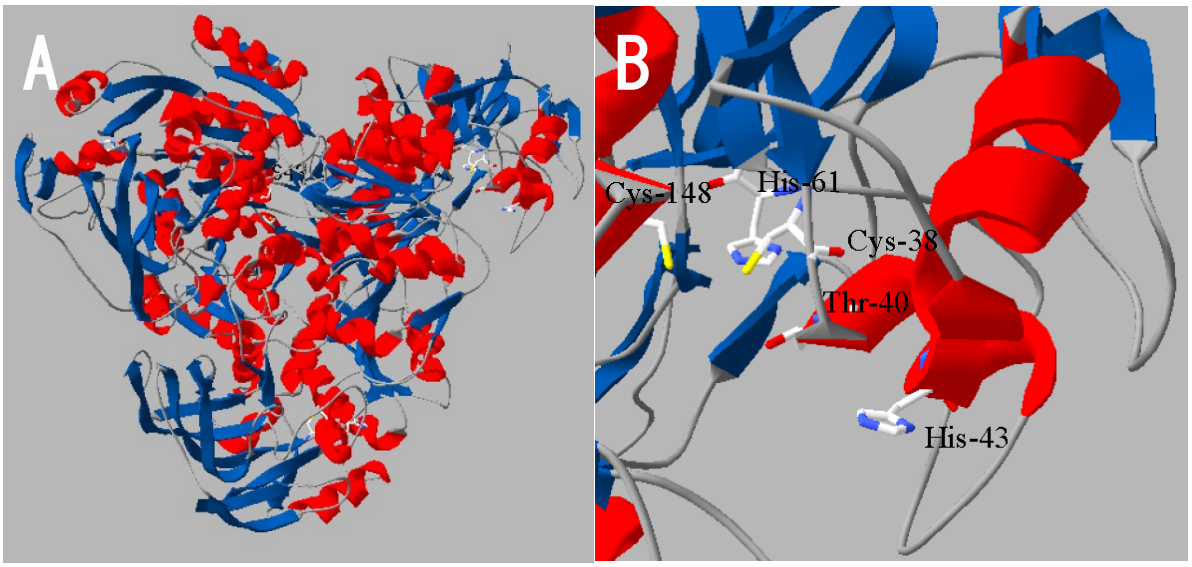


FIG. 5. Higher structure of ADH-T predicted with SWISS-MODEL. A, Tetramer structure of ADH-T: B, Detail picture of the active pocket.

Putative mechanism for the proton release system for ADH-T was composed of a zinc-bound water molecule, Thr40, and His43. The proton release is through a hydrogen -bonded network to relay the proton to solvent.

Analysis of the proton release system by amino acid substitution.

To verify the reaction mechanism of ADH-T, the putative catalytic amino acid residues, Cys38, Thr40, and His43, were substituted by site-directed mutagenesis with chemically synthesized oligonucleotides (Fig. 6). The following mutant enzymes were produced: Cys38Ser (Cys38 as a putative catalytic zinc ligand was replaced by serine), Thr40Ala, Thr40Ser, and His43Ala. Their cell lysates were used for ADH-assay and SDS-PAGE. All mutant enzymes were produced at the same level (data not shown). However, Cys38Ser, Thr40Ala, and His43Ala had no ADH-activity.

In contrast, Thr40Ser showed ADH-activity. The wild-type ADH-T and the mutant enzyme Thr40Ser were purified to homogeneity. Thr40Ser had the same pH profile as the wild-type enzyme, although the level of enzyme activity was lower (Fig. 7). These results indicate that Cys38, His43, and the hydroxyl group of Thr40 or serine residue are essential for enzyme activity and that the lower level of activity of Thr40Ser might be explained by

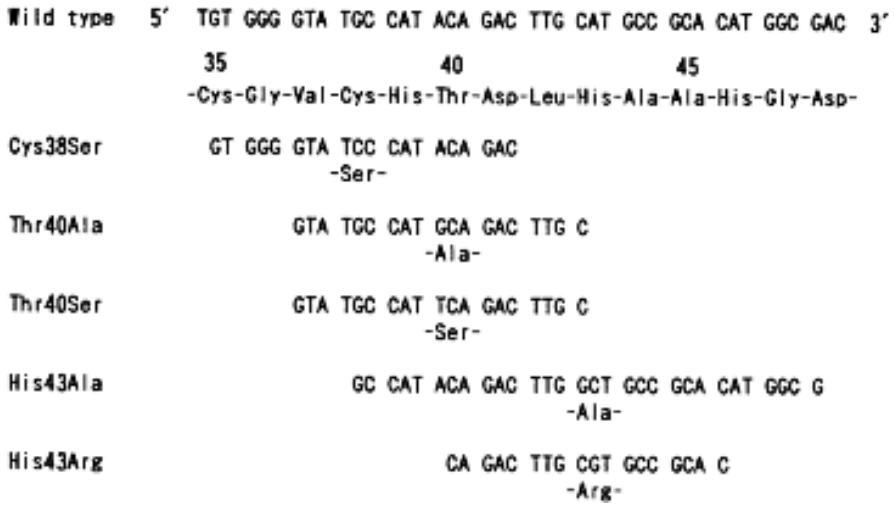


FIG. 6. Nucleotide sequence and deduced amino acid sequence of the catalytic site and its flanking regions. Synthetic oligonucleotides to introduce mutations are shown. Mutated amino acids are indicated below the nucleotides. Amino acid numbers are shown above the amino acid sequences.

a subtle change of steric conformation. The proton in the ADH-T reaction mechanism would be transported from a water molecule, through the hydroxyl group of Thr40, and released from the imidazole ring of His43 (Fig. 5B).

Change of the enzyme pH profile by site-directed mutagenesis.

His43 was substituted by arginine to alter the *pK*a of the side chain (i.e., the *pK*a of the imidazole ring of histidine is 6.0 and that of the guanidine group of arginine is 12.5). We inferred that this mutation might disturb the *pK*a of the proton release group and result in pH dependence different from that of wild-type ADH-T. The mutant enzyme His43Arg was produced and purified to homogeneity. The pH dependences of ADH-T, Thr40Ser, and His43Arg were tested by using purified enzymes. Wild-type ADH-T and Thr40Ser showed maximum activity at around pH 7.8, corresponding to the *pK*a of 7.6 of the proton release group of horse liver ADH in the presence of NAD. In contrast, His43Arg exhibited a lower level of activity under acidic conditions but a higher level of activity under alkaline conditions than the wild-type did. The maximum activity was observed at pH 9.0. Surprisingly, the maximum activity of His43Arg was about two fold higher than that of the wild-type (Fig. 7). Thus, the optimum pH of ADH-T was shifted from neutral to alkaline by replacing the catalytic amino acid His43 with arginine.

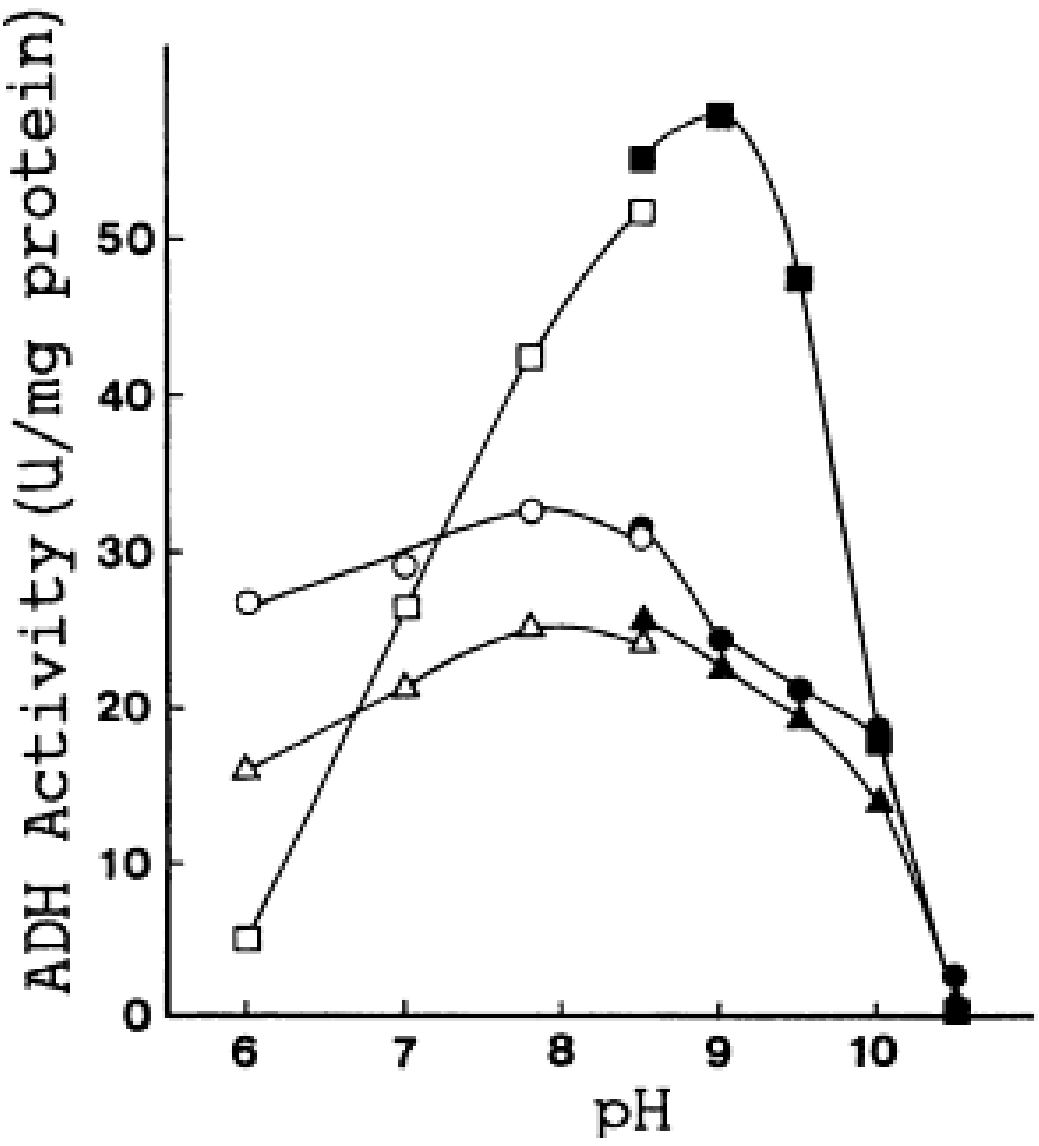


FIG. 7. pH profiles for ethanol of wild-type ADH-T (○and●), Thr40Ser mutant enzyme (△and▲), and His43Arg (□and■).

Open symbols, enzyme assay in 100 mM potassium phosphate buffer; closed symbols, enzyme assay in 100 mM glycine-KOH buffer. Enzyme activity was assayed under the standard conditions described in the text, except for buffer pH. When the NAD concentration in the reaction mixtures was reduced to 0.2 mM, nearly the same results were obtained. Therefore, 1.0 mM NAD was actually excessive at different pH conditions.

Efficient purification of thermostable enzyme

The ADH positive mutant enzymes Thr40Ser (Thr40 was substituted by serine) and His43Arg were obtained by site-directed mutagenesis. The wild-type enzyme, ADH-T (molecular weight 36,098), and the mutants, Thr40Ser and His43Arg, were easily purified to homogeneity from the transformant of *B. subtilis*, respectively (Fig. 8). Since wild-type ADH-T and two mutants were thermostable at 60°C for 1 hour (data not shown), heat-treatment was a powerful step for purification.

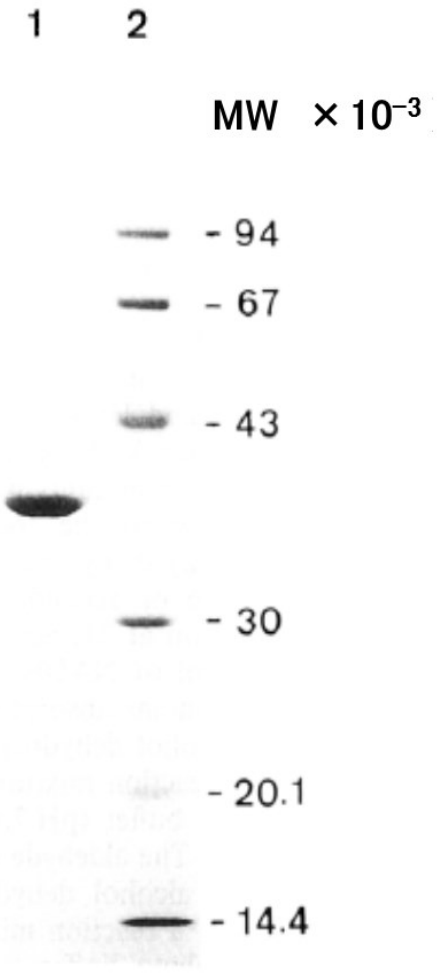


FIG. 8. SDS-PAGE photograph of purified enzyme.
Lane: 1, the purified wild-type ADH-T; 2, molecular weight markers. The mutant enzymes, Thr40Ser and His43Arg, gave the same results (photograph not shown).

Amino acid substitutions for substrate specificity of the alcohol dehydrogenase

More intensive studies have been conducted with the wild-type ADH-T and the mutant enzymes, Thr40Ser and His43Arg. It was shown that the hydroxyl group of Thr40 or Ser40 is essential for ADH-activity, and that the imidazole ring of His43 can be substituted by the guanidine group of Arg43 as a catalytic side chain. The optimum pH of Thr40Ser was 7.8, like ADH-T. While that of His43Arg in contract, shifted from 7.8 to 9.0. The substitution, His43 (*pK*a 6.0) by arginine (*pK*a 12.5), would change the *pK*a value of the catalytic group. The pH-dependence of the enzyme should be attributed to the *pK*a value of the proton release group (38). Thr40Ser exhibited lower ADH-activity than wild-type ADH-T. In contrast, the maximum activity of His43Arg showed two-fold higher activity than the wild-type. These phenomena would be explained by the conformational changes of the catalytic group.

The space available in the active pocket of ADH-T for positioning the substrate was inferred to be restricted by the side chains of Thr40 and His43 as suggested by Xray analysis with horse liver ADH (7, 9). The substrate specificity of ADH-T and its mutants was systematically tested.

Wild-type ADH-T was active not only with primary alcohols, but also with secondary alcohols. As the reverse reaction, acetaldehyde and ketones were reduced by the enzyme (Table 1). The substrate specificity of Thr40Ser was nearly identical to that of wild-type ADH-T. However, the mutant enzyme exhibited higher activity for 2-methyl-l-propanol, 3-pentanol, and cyclohexanol (Table 1). Horse liver ADH (31), having a catalytic serine corresponding to Thr40 of ADH-T, is active with cyclohexanol (7). However, yeast ADH II (30), having a catalytic threonine, is not active with cyclohexanol (7). The length of the side chain of Thr40 or Ser40 must be significant for substrate binding. The substrate specificity of Thr40Ser could be explained by the expansion of the substrate binding pocket

TABLE 1. Substrate specificity of ADH-T and its derivatives

Substrates	ADH activity (U/mg protein)		
	Wild type	Thr40Ser	His43Arg
Primary alcohols			
Methanol	0.7	ND	ND
Ethanol	32.5	25.9	41.2
1-Propanol	27.1	19.5	42.6
1-Butanol	22.2	13.7	39.5
1-Pentanol	52.5	35.1	32.8
2-Propen-1-ol	30.9	22.9	52.7
2-Methyl-1-propanol	42.9	63.4	7.0
Secondary alcohols			
2-Propanol	73.6	58.5	16.2
2-Butanol	70.1	73.1	7.9
2-Pentanol	85.8	84.6	3.8
3-Pentanol	1.8	7.9	ND
Cyclohexanol	4.5	10.7	ND
Diols, Triol			
2,4-Pentanediol	8.4	18.4	ND
2-Methyl-2,4-pentanediol	6.3	ND	ND
Glycerol	ND	ND	ND
Aldehyde			
Acetaldehyde	98.8	42.4	293.0
Ketones			
Acetone	96.6	16.1	1.4
2-Butanone	17.6	20.0	1.2
2-Pentanone	99.4	69.1	16.6
3-Pentanone	18.6	24.1	1.6
Cyclohexanone	6.8	10.5	1.5
Chiral alcohols			
R-2-Butanol	23.0	25.5	0.6
S-2-Butanol	83.9	80.5	12.4

Enzyme activity was assayed under the standard condition by using various alcohols, acetaldehyde, and ketones at the same concentration (100 mM). ND, ADH activity was not detectable (less than 0.5 U/mg protein).

by the substitution; in other words, the methyl group of Thr40 was eliminated in Ser. His43Arg exhibited higher activity for primary alcohols (except for 1-pentanol and branched alcohol such as 2-methyl-l-propanol) and acetaldehyde than wild-type ADH-T.

However, the mutant enzyme showed little activity to secondary alcohols, diols, triol, and ketones (Table 1). The drastic change in the substrate specificity of His43Arg might be

caused by a size reduction of the substrate binding pocket occupied by a long side chain of arginine residue. The preferential reaction for chiral (R or S) 2-butanol was not changed by these mutations (Thr40Ser, and His43Arg).

An alcohol dehydrogenase catalyzes the oxidation/reduction reaction between ethanol and acetaldehyde. Some isozymes are suitable for ethanol oxidation, the others for acetaldehyde reduction. The wild-type ADH-T and Thr40Ser had a low *K*m value for ethanol (*K*m-e), whereas His43Arg gave a much higher *K*m-e value. To consider the properties of the enzymes, I compared their rate constants with those of ADH I and ADH II (21) from *Saccharomyces cerevisiae* (Table 2). A constitutive enzyme, ADH I, has a high *K*m-e value, and seems to be mainly responsible for the production of ethanol from acetaldehyde in cells grown anaerobically. Another cytoplasmic isozyme, ADH II, has a much lower *K*m-e. ADH II, an inducible enzyme, is found only in aerobically grown yeast. A primary function of ADH II is the conversion of ethanol accumulated during anaerobic growth into acetaldehyde with the concomitant reduction of NAD (21, 30).

TABLE 2. Kinetic parameters of alcohol dehydrogenase for ethanol and acetaldehyde

Enzyme	Ethanol		Acetaldehyde	
	kcat-e (s^{-1})	K_m (mM)	kcat-a (s^{-1})	K_m (mM)
Wild type	40.2±0.6	0.356±0.003	87.0±3.1	0.0852±0.0013
Thr40Ser	23.6±0.7	0.203±0.004	35.9±1.9	0.0611±0.0006
His43Arg	37.6±7.6	19.3±0.3	198±13	3.69±0.03
ADH-I[a]		24		3.4
ADH-II[a]		2.7		0.045

All values were determined by Lineweaver-Burk plot (39) under the standard condition given in the text by varying the ethanol or acetaldehyde concentration.

[a] These two isozymes are cytoplasmic alcohol dehydrogenases from *S. cerevisiae* (21)

It should be able to use a low concentration of ethanol efficiently since its *K*m-e value is about 9-fold lower than that of yeast ADH I (21, 40). The wild-type ADH-T, having a smaller *K*m-e value than that of ADH II, should be powerful for ethanol oxidation, even at a low concentration. In contrast, His43Arg gave a large *K*m-e value and remarkably high *k*cat value for acetaldehyde (*k*cat-a). It would function as a fermentative isozyme, reducing acetaldehyde to ethanol like yeast ADH I. His43Arg showed its optimum activity at pH 9.0. Its *k*cat value ($71.9 \pm 2.7 s^{-1}$) toward ethanol at pH9.0 was much larger than that measured under pH 7.8, and the *K*m value (21.0 ± 0.1 mM) at pH 9.0 was similar to that under pH 7.8. However, the values for acetaldehyde at pH 9.0 could not be measured because of the high background of the reaction mixture containing acetaldehyde under the alkaline condition, pH 9.0.

DISCUSSION

The thermostable alcohol dehydrogenase (ADH-T) gene (*adhT*) from *G. stearothermophilus* was cloned in *B. subtilis*. Wild-type ADH-T and its derivatives were easily purified from the transformants to homogeneity by heat treatment and DEAE-cellulose ion-exchange chromatography. Heat treatment is a powerful step to purify thermostable enzymes as shown in Fig. 2.

By site-directed mutagenesis, some *adhT* mutants were constructed. Studies with the mutant enzymes, which were constructed on the basis of three-dimensional structure information available for horse liver ADH, provided considerable information about ADH-T catalysis. Thr40 and His43 should be essential as the active center of ADH-T. Cys38 would be a ligand of the catalytic zinc (Fig. 5B). The pH profile of ADH-T was altered by replacing the catalytic amino acid histidine, His43, with arginine. By the substitution, the *pK*a of the active group, which was composed of a water molecule, Thr40, and His43, was thought to be shifted from neutral to alkaline. As a result, the alkaline enzyme His43Arg was obtained. Under acidic conditions, the mutant enzyme exhibited a lower level of activity than did wild-type ADH-T. The explanation for this might be that substitution of His43 with arginine slowed down the proton release reaction under acidic conditions.

Generally speaking, the *pK*a value of the active center of an enzyme can influence the pH profile. In other words, the pH profile of an enzyme could be altered by changing the *pK*a value of a catalytic amino acid. For example, an active-site histidine residue of serine protease acts as a general base in enzyme catalysis, and its *pK*a rules enzyme activity. Increasing the overall negative charge on the enzyme should raise the *pK*a of the active-site histidine by stabilizing the protonated form of the histidine, whereas increasing the positive charge should lower the *pK*a by destabilizing the protonated form of the histidine. Its

activity under acidic condition increased when the number of lysine residues of the enzyme surface was increased by site-directed mutagenesis (38).

Since enzymes are proteins containing many ionizable groups, they exist in a whole series of different states of ionization. However, only one of the ionic forms of the active center is catalytically active (41, 42). This experiment shows that the *pK*a value of an active site is responsible for the pH profile of an enzyme and that the optimum pH is altered by substituting a catalytic amino acid.

The alcohol dehydrogenase, involving an arginine residue in the proton release system, has never been isolated from any organism and tissue, nor has it been constructed except for this work. The substitution of catalytic histidine residue by arginine residue in other alcohol dehydrogenases would change their properties, and useful and powerful enzymes might be created. The activity of the His43Arg mutant enzyme at its optimum pH of 9.0 is about twice that of the wild-type at pH 7.8. Arg43 rather than His43 might be more sterically suitable for proton transfer from Thr40. The level of ADH-activity of Thr40Ser at its optimum pH of 7.8 is lower than that of the wild-type, perhaps because of steric hindrance.

SUMMARY

Using *B. subtilis* as a host and pTB524 as a vector plasmid, we cloned the thermostable alcohol dehydrogenase (ADH-T) gene (*adhT*) from *G. stearothermophilus* NCA1503 and determined its nucleotide sequence. The deduced amino acid sequence (337 amino acids) was compared with the sequences of ADHs from different origins. The amino acid residues responsible for the catalytic activity of horse liver ADH had been clarified on the basis of three-dimensional structure. Since those catalytic amino acid residues were fairly conserved in ADH-T and other ADHs, ADH-T was inferred to have basically the same catalytic mechanism (a proton release system) as horse liver ADH. The putative proton release system of ADH-T was elucidated by introducing point mutations at the catalytic amino acid residues, Cys38, Thr40, and His43, with site-directed mutagenesis. Cys38Ser (Cys38 was replaced by serine), Thr40Ala, and His43Ala had no ADH-activity. However, the mutant enzyme Thr40Ser showed a little lower level of activity than wild-type ADH-T did. These results indicate that the hydroxyl group of serine instead of threonine can also be used for the catalytic activity. Catalysis of the thermostable alcohol dehydrogenase from *G. stearothermophilus* is performed by the proton release system involving a zinc-bound water molecule, a hydroxyl group of Thr40, and an imidazole ring of His43.

The mutant enzyme, Thr40Ser, had a tendency toward lower activity for primary alcohols than the wild-type enzyme. However, the mutant enzyme became more active for substrates with a larger side chain, such as 2-methyl-l-propanol and cyclohexanol. This phenomenon might be explained by the fact that the methyl group of Thr40 was eliminated in serine residue.

To change the *pK*a value of the putative system, His43 was replaced by the more basic amino acid arginine residue. As a result, the optimum pH of the mutant enzyme His43Arg was shifted from 7.8 (wild-type enzyme) to 9.0. His43Arg exhibited a higher level of activity

than wild-type enzyme at the optimum pH. His43Arg exhibited higher activity to primary alcohols (except 2-methyl-1-propanol) and acetaldehyde (as a reverse reaction) than the wild-type ADH-T, but little activity for secondary alcohols and ketones. The *K*m value for ethanol (*K*m-e) of His43Arg was fifty-fold larger than that of the wild-type enzyme.

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CHAPTER 2

Efficient Expression of the Gene Coding for Thermostable Aldehyde Dehydrogenase from *Geobacillus stearothermophilus*, and Characterization of the Enzyme

INTRODUCTION

Aldehyde dehydrogenase (ALDH) is a ubiquitous enzyme which participates in alcohol metabolism and the biological oxidation of aldehyde compounds. In higher eukaryotes, at least two isozymes of ALDH, one cytoplasmic and the other mitochondrial, have been identified and shown to differ in their enzymatic properties, including *K*m values for coenzyme (NAD or NADP) and acetaldehyde, response to inhibitors, and substrate specificity (1-3). The reports for ALDH primary structures deduced from DNA sequence analyses from yeast (4), mammals (5), *Escherichia coli* (6), and other organisms (7-9) led to the prediction of important amino acid residues. Out of these residues, cysteine and glutamate at the middle of the amino acid sequence of ALDH, were extraordinarily conserved (4, 9), suggesting that they are catalytic amino acid residues. However, the catalytic mechanism of ALDH, which has been mainly investigated using animal liver ALDH, was not completely elucidated (10, 11).

Generally speaking, ALDH is very unstable because of the natural oxidation. Therefore, it is considerably difficult to analyze the characteristics of the enzyme, and most of the properties of the enzyme are still unknown. By the way, thermostable enzymes were shown to be superior in stability as described in chapter 1. Thermostable ALDH (ALDH-T) from *Geobacillus stearothermophilus* would be stable and useful for understanding the catalytic mechanism of ALDH. In this chapter, the molecular cloning and nucleotide sequence of the *aldhT* gene coding ALDH-T from *G. stearothermophilus* are described. I

also report here an efficient production of ALDH-T in *E. coli,* and purification and characterization of the enzyme. Since the *adhT* gene coding thermostable alcohol dehydrogenase (ADH-T) has already been cloned from *G. stearothermophilus* (12) and described in chapter 1 of this thesis, further knowledge about both ADH and ALDH would help clarify the mechanism of alcohol metabolism in thermophilic bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed as follows. *G. stearothermophilus* SIC1 was used as a DNA donor. *E. coli* JM109 and plasmid pUC19 (13) were used to construct the gene bank of *G. stearothermophilus* SIC1. *E. coli* TG1 and M13 mpl8 or mpl9 were used to subclone DNA fragments for nucleotide sequencing. A low-copy-number plasmid, pTB523 (14), was used as a vector plasmid for *Bacillus subtilis* MIll3 and *G. stearothermophilus* SIC1 (16).

E. coli and *B. subtilis* MIll3 were grown at 37°C in L-broth (10 g/l Bacto-tryptone, 5 g/l yeast extract, and 5 g/l NaCl, adjusted to pH 7.3). *G. stearothermophilus* SIC1 was grown at 55°C in 2L-broth (20 g/l Bacto-tryptone, 10 g/l yeast extract, and 5 g/l NaCl, adjusted to pH 7.3) (15). Tetracycline was added to the culture broth for transformants of *B. subtilis* (25 μg/ml) and *G. stearothermophilus* (5 μg/ml), respectively. Transformants of *E. coli* with pUC19 or its derivatives were grown in L-Ap broth containing 50 μg/ml of ampicillin. M9 broth (17) was used as a minimal medium for *E. coli.* Agar plates were solidified with 20 g/l and 15 g/l agar for cell growth at 55°C and 37°C, respectively.

DNA manipulation

Plasmids were extracted by either rapid alkaline extraction or CsCl-ethidium bromide

equilibrium density gradient ultracentrifugation (18). Chromosomal DNA was prepared from a sarcosyl lysate of cells as described previously (19). Restriction endonucleases, and T4 DNA ligase (Toyobo Co., Osaka) were handled according to the manufacturer's instructions.

Transformation

Transformation of *E. coli* with plasmid DNA was conducted according to the method of Hanahan (20). Transformation of competent *B. subtilis* cells was performed as previously described (21), while the transformation of *G. stearothermophilus* was carried out using the protoplast procedure described previously (22).

Construction of a gene library from *G. stearothermophilus* and library screening

The purified chromosomal DNA was digested with restriction endonuclease and ligated to digested pUC19 using T4 DNA ligase. To construct a gene library, *E. coli* JM109 was transformed with the resulting ligation mixture, and selected on L-Ap agar containing 47.6 μg/ml of isopropyl-β-D-thio-galactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO, USA) and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-,-D-galactoside (X-gal) (Sigma). White colonies among the transformants were selected, screened by colony hybridization, grown on L-Ap agar at 37°C for 5 hours, and replicated onto a nylon membrane (Amersham, Buckinghamshire. UK) (23). Colony hybridization was performed according to the manufacturer's instructions.

Nucleotide sequencing

Nucleotide sequencing was carried out according to the dideoxynucleotide chain terminating method of Sanger et al. (24), using the Sequenase sequencing kit (United States

Biochemical Co., Cleveland, OH, USA). Single-stranded DNA from subclones was prepared using M13 phage. Chemically synthesized oligonucleotide was used as an inner probe when necessary.

Enzyme purification

E. coli JM109 cells carrying the ALDH-positive recombinant plasmid, pUALD27R, were grown in L-broth containing 50 μg/ml of ampicillin and then harvested by centrifugation (10,000×g, 5 min). All subsequent procedures were carried out at 4°C unless otherwise stated. Cells were washed with 50 mM Tris-HC1 buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and 50 mM KCI, suspended in the same buffer (1/10 volume of original culture broth) and disrupted by sonication. The sonicated suspension was centrifuged (15,000×g, 30 min) to obtain a supernatant which was used as a cell-free extract. The cell-free extract was heated at 60°C for 10 min in order to separate the thermostable ALDH-T from other *E. coli* proteins. The heat-denatured proteins were removed by centrifugation (20,000×g, 30 min). The supernatant was saturated to 30% with ammonium sulfate by adding solid ammonium sulfate and allowing the mixture to stand for 4 hours. After centrifugation (15,000×g, 30 min), ammonium sulfate was added to the supernatant to achieve 60% saturation, and the solution was kept at 4°C overnight. The precipitate was collected by centrifugation (15,000×g, 30 min), dissolved in a minimum volume of 50 mM Tris-HCI buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and 100 mM KC1, and dialyzed against the same buffer. The enzyme sample was applied to a Toyopearl HW55 gel column (Tosoh Corp., Tokyo) that had been equilibrated with the above mentioned buffer. The enzyme was eluted with the same buffer and then fractionated. ALDH fractions were pooled and applied to a DEAE-BioGel A (Bio-Rad, Richmond, CA, USA) column that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing

10 mM 2-mercaptoethanol. Elution of the enzyme was performed with a linear gradient of potassium chloride (0 - 0.5 M) in the same buffer. The active fractions containing ALDH-T were pooled. ALDH-T was stable within the pH range of 7 to 9. Buffer with a pH of 7.8 was used throughout the enzyme purification procedures.

Assay of ALDH-activity and protein concentration

ALDH-activity was measured spectrophotometrically by tracing NADH formation at 340 nm. ALDH-activity was expressed as μ mole of NADH produced per min, with a molar coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$. The standard assay was performed at 55℃ in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.8), I mM NAD and 0.1 M acetaldehyde. Substrate inhibition was observed at a high acetaldehyde concentration (1.0 M), but not at 0.1 M. Reaction was started by adding the enzyme solution to the prewarmed reaction mixture, and absorbance at 340 nm was monitored. Protein concentration was determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, USA), using bovine serum albumin as a standard protein.

Steady-state enzyme kinetics

The Michaelis constant and maximum activity of the enzyme were determined by extrapolation to zero-order condition with respect to each substrate (aldehydes) as described by Dalziel (25). This was accomplished by drawing a Lineweaver-Burk plot (26) with a fixed concentration of one substrate, which was then varied to obtain several lines. Two kinds of secondary plot were produced from this data. The first is a plot of the ordinate intercept versus the reciprocals of fixed substrate concentration, while the second is a plot of the slope versus the reciprocals of a fixed substrate concentration. All of the kinetic constants were evaluated from the secondary plot. Since the lag phase occurred in the early

stage of the enzyme reaction, the initial velocities were measured at a linear portion of the increase in absorbance at 340 nm which was maintained for several minutes after the lag was over.

Estimation of molecular weight

The molecular weight of native ALDH-T was estimated by gel filtration on Fast Protein Liquid Chromatography (FPLC) using a 12HR 10/30 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated and the proteins were eluted with 50 mM Tris-HC1 (pH 7.8), 10 mM 2-mercaptoethanol, and 100 mM KCI at a flow rate of 12 ml/h. The enzyme was detected by absorption at 280 nm and by assaying ALDH-activity. Standard proteins used for calibration were cyanocobalamin (MW 1,350), myoglobin (MW 17,000), ovalbumin (MW 44,000), bovine serum albumin (MW 66,000), aldolase (MW 158,000), catalase (MW 232,000), ferritin (MW 440,000), and thyroglobulin (MW 669,000). Blue dextran (MW 2,000,000) was used to determine the void volume. The subunit molecular weight of ALDH-T was estimated by polyacrylamide (7.5%) gel electrophoresis under denaturing conditions in the presence of sodium dodecylsulfate (SDS-PAGE) as described by Laemmli (27).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D13846, and D14575.

RESULTS

Cloning of the gene coding for aldehyde dehydrogenase

A highly conserved amino acid sequence would be the most useful candidate as a probe for colony hybridization. Highly conserved regions containing putative catalytic amino acids (cysteine and glutamate at the middle of the amino acid sequence of ALDH) were proposed by comparing with published ALDH sequences. Two types of mix-oligonucleotides corresponding to the above amino acid sequences were synthesized for gene cloning. These oligonucleotides were used as a probe for Southern hybridization against each restriction enzyme-digest of *G. stearothermophilus* SIC1 chromosomal DNA. An *Eco*RI-digest of about 2.5 kb was intensely hybridized with one of the probes, which corresponds to the amino acid sequence containing the glutamate. The 2.5 kb *Eco*RI fragment was cloned in *E. coli* JM109 using colony hybridization. The recombinant plasmid was named pUALD25. Various fragments were subcloned in Ml3 mpl8 and mp19 phages in order to sequence both strands. Nucleotide sequence analysis revealed that the DNA fragment lacked a sequence coding for the C-terminal portion of ALDH. Colony hybridization was performed using ALDH coding region as a probe.

Nucleotide sequence of the *aldhT* gene

The nucleotide sequence of the *aldhT* gene and the flanking regions is shown in Fig. 1. A large open reading frame (ORF) was observed, composed of 1,464 bp corresponding to 488 amino acid residues. The molecular weight was estimated to be 52,912. A Shine-Dalgarno (SD) sequence, a probable ribosome binding site, was located 9 bp upstream from the initiation codon (ATG). A typical prokaryotic promoter sequence (28, 29) was located 33 bp upstream from the initiation codon. A nucleotide sequence resembling typical prokaryotic terminators was found downstream from the ORF. And

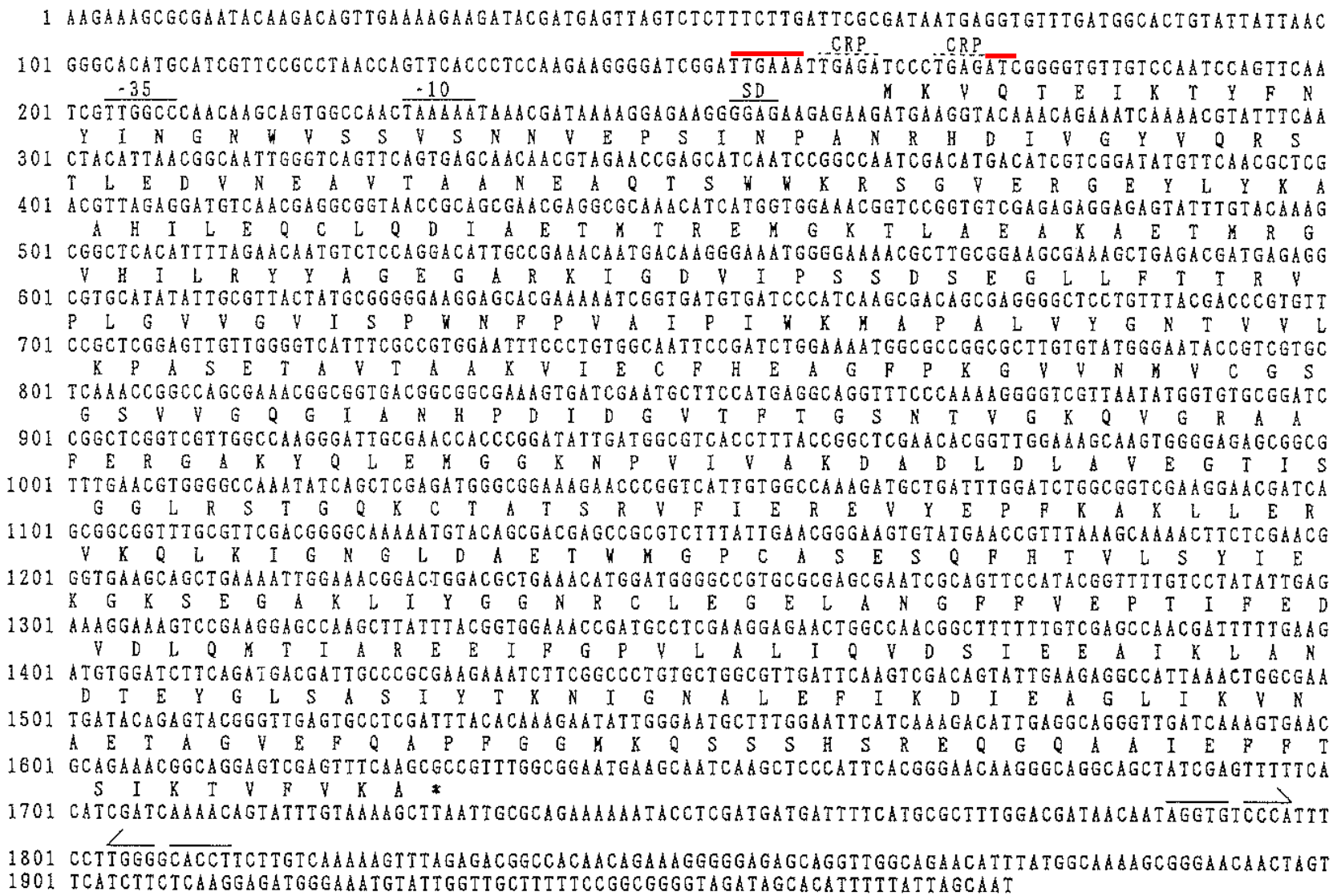
another promoter sequence resembling the consensus sequence of sigma 32 promoter (30) was found at the 5'-flanking region of the ORF.

The deduced amino acid sequence of the protein coded in this ORF exhibited significant homology (identity 36%) with *Gluconacetobacter europaeus* (31), 38% with *Acetobacter pasteurianus* (32), 47% with *Saccharomyces cerevisiae* (33), 43% with human (34), 42% with horse (35), 30% with *Thermococcus kodakaraensis* (36) (Fig. 2). The catalytic amino acids, Glu255 (glutamic acid position at 255), Cys289, were fairly conserved.

The overall reaction mechanism of the ALDH-T would be three main steps as mentioned by crystallography study of different ALDHs: (i) nucleophilic attack of the thiol group of the catalytic cysteine, Cys289, on the carbonyl carbon of the aldehyde substrate; (ii) hydride transfer from the tetrahedral thiohemiacetal intermediate to the pyridine ring of NAD(P)+; and (iii) hydrolysis of the resulting thioester intermediate (deacylation). The catalytic glutamate, Glu55, would be the general base that activates the hydrolytic water molecule in the deacylation step (37).

→

FIG. 1. Nucleotide sequence of the *aldhT* gene and the deduced amino acid sequence of the coded protein.
A probable Shine-Dalgarno (SD) sequence and promoter sequence are indicated by solid lines. The speculated promoter recognized with sigma 32 is indicated by red lines. The sequences resembling the cyclic AMP receptor protein (CRP) binding site are also shown by dotted lines. The inverted repeat of the terminator is shown by an arrow. The amino acid sequence (one-letter code) is shown above the nucleotide sequence. An asterisk indicates a stop codon.


CRP
CRP
-35
-10
SD
1 AAGAAAGCGCGAATACAAGACAGTTGAAAAGAAGATACGATGAGTTAGTCTCTTTCTTGATTCGCGATAATGAGGTGTTTGATGGCACTGTATTATTAAC
101 GGGCACATGCATCGTTCCGCCTAACCAGTTCACCCTCCAAGAAGGGGATCGGATTGAAATTGAGATCCCTGAGATCGGGGTGTTGTCCAATCCAGTTCAA
M K V Q T E I K T Y F N
201 TCGTTGGCCCAACAAGCAGTGGCCAACTAAAAATAAACGATAAAAGGAGAAGGGGAGAAGAGAAGATGAAGGTACAAACAGAAATCAAAACGTATTTCAA
Y I N G N W V S S V S N N V E P S I N P A N R H D I V G Y V Q R S
301 CTACATTAACGGCAATTGGGTCAGTTCAGTGAGCAACAACGTAGAACCGAGCATCAATCCGGCCAATCGACATGACATCGTCGGATATGTTCAACGCTCG
T L E D V N E A V T A A N E A Q T S W W K R S G V E R G E Y L Y K A
401 ACGTTAGAGGATGTCAACGAGGCGGTAACCGCAGCGAACGAGGCGCAAACATCATGGTGGAAACGGTCCGGTGTCGAGAGAGGAGAGTATTTGTACAAAG
A H I L E Q C L Q D I A E T M T R E M G K T L A E A K A E T M R G
501 CGGCTCACATTTTAGAACAATGTCTCCAGGACATTGCCGAAACAATGACAAGGGAAATGGGGAAAACGCTTGCGGAAGCGAAAGCTGAGACGATGAGAGG
V H I L R Y Y A G E G A R K I G D V I P S S D S E G L L F T T R V
601 CGTGCATATATTGCGTTACTATGCGGGGGAAGGAGCACGAAAAATCGGTGATGTGATCCCATCAAGCGACAGCGAGGGGCTCCTGTTTACGACCCGTGTT
P L G V V G V I S P W N F P V A I P I W K M A P A L V Y G N T V V L
701 CCGCTCGGAGTTGTTGGGGTCATTTCGCCGTGGAATTTCCCTGTGGCAATTCCGATCTGGAAAATGGCGCCGGCGCTTGTGTATGGGAATACCGTCGTGC
K P A S E T A V T A A K V I E C F H E A G F P K G V V N M V C G S
801 TCAAACCGGCCAGCGAAACGGCGGTGACGGCGGCGAAAGTGATCGAATGCTTCCATGAGGCAGGTTTCCCAAAAGGGGTCGTTAATATGGTGTGCGGATC
G S V V G Q G I A N H P D I D G V T F T G S N T V G K Q V G R A A
901 CGGCTCGGTCGTTGGCCAAGGGATTGCGAACCACCCGGATATTGATGGCGTCACCTTTACCGGCTCGAACACGGTTGGAAAGCAAGTGGGGAGAGCGGCG
F E R G A K Y Q L E M G G K N P V I V A K D A D L D L A V E G T I S
1001 TTTGAACGTGGGGCCAAATATCAGCTCGAGATGGGCGGAAAGAACCCGGTCATTGTGGCCAAAGATGCTGATTTGGATCTGGCGGTCGAAGGAACGATCA
G G L R S T G Q K C T A T S R V F I E R E V Y E P F K A K L L E R
1101 GCGGCGGTTTGCGTTCGACGGGGCAAAAATGTACAGCGACGAGCCGCGTCTTTATTGAACGGGAAGTGTATGAACCGTTTAAAGCAAAACTTCTCGAACG
V K Q L K I G N G L D A E T W M G P C A S E S Q F H T V L S Y I E
1201 GGTGAAGCAGCTGAAAATTGGAAACGGACTGGACGCTGAAACATGGATGGGGCCGTGCGCGAGCGAATCGCAGTTCCATACGGTTTTGTCCTATATTGAG
K G K S E G A K L I Y G G N R C L E G E L A N G F F V E P T I F E D
1301 AAAGGAAAGTCCGAAGGAGCCAAGCTTATTTACGGTGGAAACCGATGCCTCGAAGGAGAACTGGCCAACGGCTTTTTTGTCGAGCCAACGATTTTTGAAG
V D L Q M T I A R E E I F G P V L A L I Q V D S I E E A I K L A N
1401 ATGTGGATCTTCAGATGACGATTGCCCGCGAAGAAATCTTCGGCCCTGTGCTGGCGTTGATTCAAGTCGACAGTATTGAAGAGGCCATTAAACTGGCGAA
D T E Y G L S A S I Y T K N I G N A L E F I K D I E A G L I K V N
1501 TGATACAGAGTACGGGTTGAGTGCCTCGATTTACACAAAGAATATTGGGAATGCTTTGGAATTCATCAAAGACATTGAGGCAGGGTTGATCAAAGTGAAC
A E T A G V E F Q A P F G G M K Q S S S H S R E Q G Q A A I E F F T
1601 GCAGAAACGGCAGGAGTCGAGTTTCAAGCGCCGTTTGGCGGAATGAAGCAATCAAGCTCCCATTCACGGGAACAAGGGCAGGCAGCTATCGAGTTTTTCA
S I K T V F V K A *
1701 CATCGATCAAAACAGTATTTGTAAAAGCTTAATTGCGCAGAAAAAATACCTCGATGATGATTTTCATGCGCTTTGGACGATAACAATAGGTGTCCCATTT
1801 CCTTGGGGCACCTTCTTGTCAAAAAGTTTAGAGACGGCCACAACAGAAAGGGGGAGAGCAGGTTGGCAGAACATTTATGGCAAAAGCGGGAACAACTAGT
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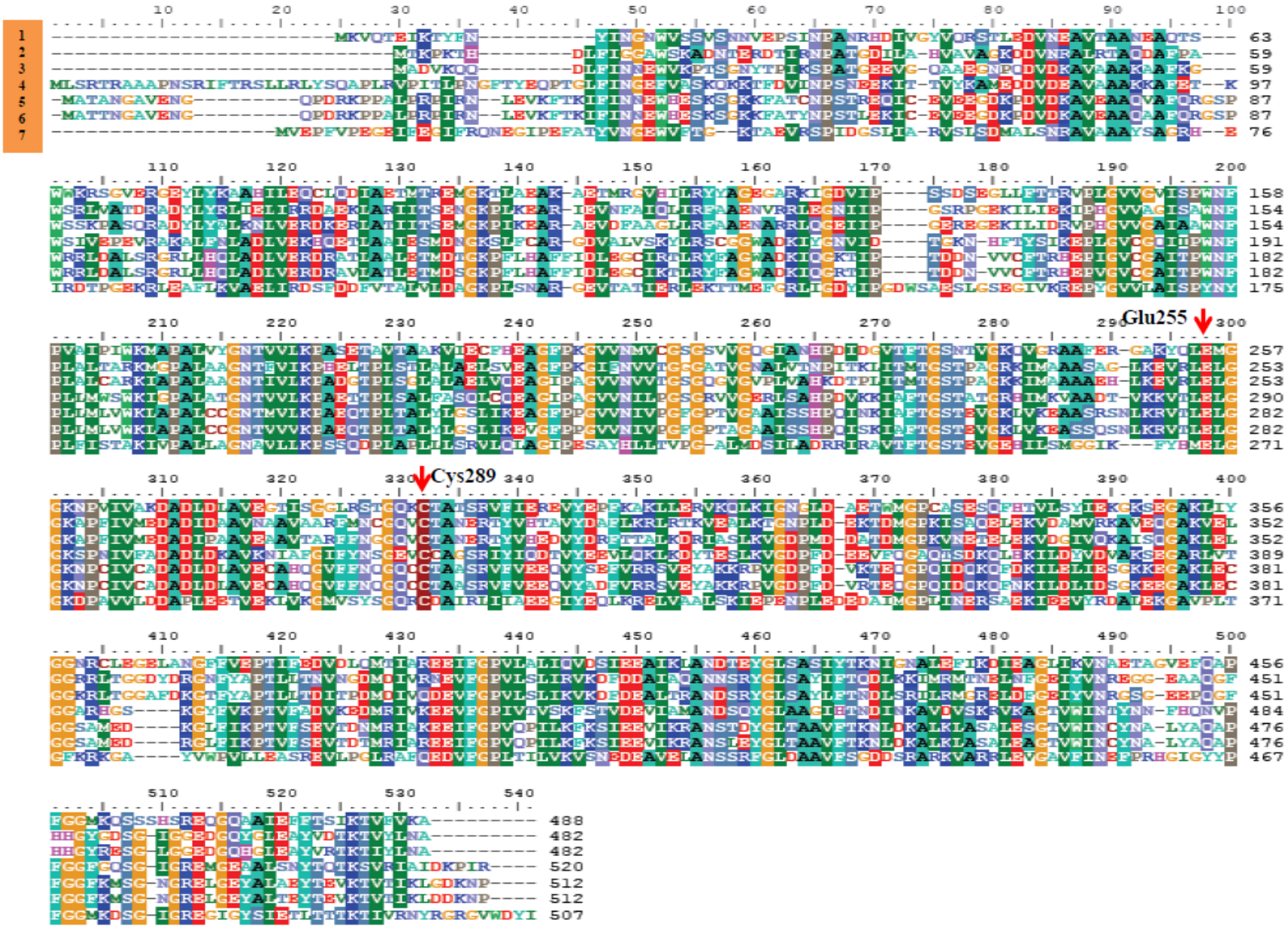
Glu255
Cys289

FIG. 2. Comparison of amino acid sequences of seven different ALDHs
Lanes: 1, *G. stearothermophilus*; 2, *G. europaeus*; 3, *A. pasteurianus*; 4, *S. cerevisiae;* 5, human; 6, horse; 7, *T. kodakaraensis*. The catalytic amino acids are indicated by arrows. ←

Expression of the *aldhT gene* in *E. coli* and *G. stearothermophilus*

A transformant of *E. coli* JMl09 carrying pUALD27R produced a large amount of ALDH-T. Even though the *aldhT* gene was inserted downstream to the pUC19 *lac* promoter, gene expression was not induced by isopropyl-, β -D-thio-galactopyranoside (IPTG). The *aldhT* gene seems to be expressed using an original promoter. *E. coli* JMl09 carrying pUALD27R was cultured at 37°C for 16 hours in minimal medium containing 0.2% glucose or ethanol as the sole carbon source. The ALDH-activities of the cell-free extracts are shown in Table 1. The transformant grown in the ethanol minimal medium exhibited 10-fold higher activity (10.5 U/mg protein) than that in glucose minimal medium (1.1 U/mg protein). , and 2-fold higher than that in L-broth (5.0 U/mg protein). The *E. coli* transcriptional factor sigma 32 might promote transcription of *aldhT* gene from *G. stearothermophilus* to survive cell at high ethanol condition.

G. stearothermophilus SIC1 showed very low ALDH-activity. To examine the *aldhT* expression in *G. stearothermophilus* SIC1, a low copy number plasmid-vector, pTB523, was used to transform the thermophile. The recombinant plasmid, pTBALD27, carrying the

TABLE 1 Effect of carbon sources on ALDH-T production in *E. coli* JM109 and its transformant

Carbon source or culture broth	ALDH activity (U/mg protein) in:	
	Host cell	Transformant
Glucose	0.006	1.1
Ethanol	0.019	10.5
L-broth	0.008	5.0

entire *aldhT* gene, was constructed. *G. stearothermophilus* with pTBALD27 was cultured in 2L-broth at 55°C. Cell-free extract of the transformant exhibited maximum activity (0.35 U/mg protein) in the early stationary phase. However, the transformant expressed lower enzyme activity than did the *E. coli* transformant.

Purification of ALDH-T

ALDH-T was purified from the transformant of *E. coli* according to the procedure described in Materials and Methods. Throughout the purification, heat treatment was quite efficient for the enrichment of the thermostable enzyme from other cellular proteins, as mentioned in a previous study (12). Aldehyde dehydrogenase from the host cell was also eliminated by the heat treatment. The ion-exchange chromatography elution pattern revealed that the highest protein peak corresponded to that of ALDH-activity, and that ALDH-T was eluted at an ionic strength of approximately 120-150 mM KCI. SDS-PAGE analysis of the final preparation showed only a single band (molecular weight 53,000), which was in good agreement with the value deduced from the *aldhT* gene sequence (Fig. 1). Approximately 100 mg of the enzyme were obtained from 1,000 mg protein in the cell-free extract. The specific activity of the purified enzyme was 36.3 U/mg protein under the standard assay condition. In general, ALDH has been reported to be a very unstable enzyme. It easily undergoes oxidation and loses activity. The oxidation of ALDH-T was prevented by adding reducing reagents such as 2-mercaptoethanol and dithiothreitol. Cell-free extract, when prepared in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM 2-mercaptoethanol, lost 40 to 60% of its ALDH-T activity upon storage at 4°C for 24 hours. By increasing the concentration of 2-mercaptoethanol to 10 mM, ALDH-T retained its original activity for a week.

Estimation of molecular weight

The native molecular weight of ALDH-T was estimated by FPLC gel filtration. ALDH-T eluted just after catalase (MW 232,000), in a fraction corresponding to a calculated molecular weight of 220,000. SDS-PAGE analysis suggested only one size of subunit, whose molecular weight was estimated to be 53,000 in the enzyme fraction (photograph not shown). Thus, ALDH-T is concluded to be composed of four identical subunits.

Effect of temperature on the activity and stability of ALDH-T

The effects of temperature on enzyme activity were tested. The optimum temperature was around 55 to 60°C (Fig. 3A). To determine the thermostability of the enzyme, the purified enzyme was incubated for 30 min at various temperatures and then cooled immediately on ice. The activity remaining at 75°C was 50 %, while no activity was observed after heating at 90°C. The enzyme was quite stable up to 65°C (Fig. 3B).

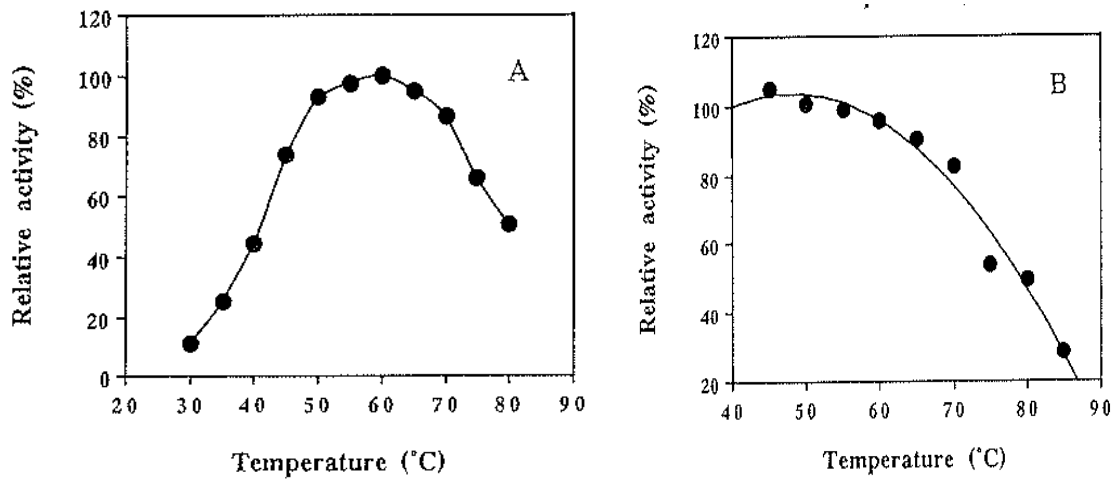

FIG. 3. (A) Effect of temperature on ALDH-T relative activity. The assay was performed at various temperatures for 10 min. (B) Heat stability of ALDH-T. The enzyme was heated at various temperatures for 30 min, and the remaining activity was assayed under the standard conditions.

Substrate specificity and kinetic constants of ALDH-T

The plots of initial velocity versus substrate (both for NAD and aldehyde) concentration revealed Michaelis-Menten type hyperbolic curves, except for very high concentrations of aldehyde. The steady-state kinetic constants were determined for various aldehydes (Table 2). Aliphatic aldehydes were excellent substrates for ALDH-T. However, ALDH-T could not oxidize benzaldehyde, an aromatic aldehyde. It is interesting to note that ALDH-T exhibited the greatest activity toward a C6-aliphatic aldehyde, hexanal. Also, the dialdehyde glutaldehyde was a poor substrate compared with the monoaldehyde n-valeraldehyde, even though the carbon chain lengths (C5) are the same (Table 2). ALDH-T required either NAD or NADP as a coenzyme. When coenzyme A was added to the standard reaction mixture with purified ALDH-T, little increase in activity was observed. Thus, it is unlikely that coenzyme A is involved in the reaction.

TABLE 2 Substrate specificity of ALDH-T[a]

Substrate	K_m (mM)	V_m (relative value)
Acetaldehyde	0.520	100
Propionaldehyde	0.548	93
Isobutylaldehyde	0.284	52
n-Valeraldehyde	0.349	64
Glutaldehyde	0.620	2
Hexanal	0.251	127
Benzaldehyde	ND	ND
NAD	0.256	114

[a] Michaelis constants (K_m) and maximum velocities (V_m) were determined using NAD and/or acetaldehyde as standard substrates. Each V_m value is shown by the relative value, taking the maximum velocity for acetaldehyde as 100. The specific activity of the purified ALDH-T is 36.3 U/mg protein. ND, ALDH activity was not detectable.

DISCUSSION

Thermostable aldehyde dehydrogenase (ALDH-T) gene from *G. stearothermophilus* was cloned and expressed highly in *E. coli.* Sequencing analysis showed that the *aldhT* gene was composed of 1,464 bp, corresponding to 488 amino acid residues, and had a calculated molecular weight of 52,912. ALDH-T was purified from the *E. coli* transformant to homogeneity, and characterized. ALDH-T appears to be a homo-tetrameric enzyme with a molecular weight of about 220,000. It is known that the quaternary structure of ALDH varies depending on the biological source, ranging from dimeric form in tumor associated ALDH (3) and spinich betaine-ALDH (9), to the more common tetrameric form. ALDH-T exhibited excellent activity toward aliphatic aldehydes; however, little activity was observed toward benzaldehyde and glutaldehyde, unlike animal ALDHs which exhibit broad aldehyde specificity (1). ALDH-T may have a narrow active site which can only accept an aliphatic carbon chain without an additional aldehyde group on the chain. The *aldhT* gene was thought to be expressed in *E. coli* using its own promoter. In the control of catabolite repression of *E. coli,* the cAMP-CRP complex can bind to the promoter and exhibit positive transcriptional control (38). Nucleotide sequences resembling the consensus sequence of the cyclic AMP receptor protein (CRP)-binding site (TGTGA) were found at the 5'-flanking region of the *aldhT* gene (Fig. 1), and may be functional in *E. coli.* The active site of ALDH has not yet been clarified. Aldehyde oxidation with ALDH has been investigated using liver ALDH. The catalytic mechanism of ALDH is thought to involve a specific cysteine residue which forms a covalent intermediate with aldehyde substrates (11, 39). Some cysteine residues in the active site of ALDH have been investigated by analyses using affinity reagents for ALDH. Cys289 is the best candidate for the active site since it is conserved in all known ALDHs, including ALDH-T (Fig. 2). The environmental amino acid sequence surrounding Cys89 has been proposed to play an

important role in substrate specificity (9, 11). The glutamate residue of human ALDH isozymes, corresponding to Glu255 of ALDH-T, has been shown to be indispensable for enzyme activity (10). Glu255 is also highly conserved in all known ALDHs. The amino acid at position 480 of ALDH-T is serine residue, not glutamate, even though a glutamate residue at this position was reported to be essential for human ALDH-activity (5). The coenzyme-binding site was suggested to exist in the C-terminal portion of the enzyme (40). There are well conserved glycine and proline residues in the C-terminal portion (Fig. 2). A study based on crystallographic analysis has not yet been performed since *G. stearothermophilus* produces only a little enzyme protein. In this study, a large amount of ALDH-T was obtained from the *E. coli* transformant. Crystallographic analysis of the thermostable enzyme would be helpful to clarify the catalytic mechanism of ALDH.

The putative gene coding for a subunit of the respiratory NADH dehydrogenase from *Bacillus stearothermophilus* was cloned in *E. coli* and the nucleotide sequence was determined. The respiratory NADH dehydrogenase catalyzes the transfer of electrons from NADH, which is generated mainly by glycolysis, the tricarboxylic acid cycle and the alcohol metabolism, to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. The electron is passed ultimately to molecular oxygen, and ATP is synthesized *via* oxidative phosphorylation. The enzyme therefore links the major catabolic and energy-producing pathways in the cell (41).

SUMMARY

The *aldhT* gene coding thermostable aldehyde dehydrogenase (ALDH-T) from *G. stearothermophilus* SIC1 was cloned by colony hybridization using *E. coli* JM109 and pUC19 as a host-vector system. Nucleotide sequence analysis showed that the *aldhT* gene was composed of 1,464 bp (488 amino acid residues) which can encode a protein with molecular weight of 52,912. The *aldhT* gene was highly expressed in *E. coli* carrying the recombinant plasmid, pUALD27R, which contains a native promoter of the *aldhT* gene. The enzyme accumulated as a soluble form and not an inclusion body in E. *coli,* and the concentration was about 10% of total cytoplasmic protein. ALDH-T was purified to homogeneity with a specific activity of 36.3 U/mg protein by heat treatment of the cell extract, ammonium sulfate precipitation, gel filtration and finally ionexchange chromatography. The molecular weight of the native ALDH-T was estimated to be 220,000 by gel filtration, whereas subunit molecular weight determined by SDS-PAGE was 53,000, suggesting a tetrameric enzyme structure. ALDH-T was considerably stable after heating at 65°C for 30 min, and the optimum temperature for the enzyme reaction was 55 to 60°C. The enzyme was capable of oxidizing several aliphatic aldehydes, particularly C6-aliphatic aldehyde and hexanal, but did not oxidize benzaldehyde, an aromatic aldehyde. The deduced amino acid sequence of ALDH-T exhibited 43 % homogeneity to that of the human cytoplasmic aldehyde dehydrogenase sequence. Two amino acid residues believed to be implicated in the active site, Cys289 and Glu255, were conserved among the different aldehyde dehydrogenases.

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CHAPTER 3

A New Way of Stabilizing Recombinant Plasmids

INTRODUCTION

A host-plasmid system of *Escherichia coli* that would warrant an industrial production of L-tryptophan by fermentation has been established (1-4). It was considered in the process that plasmid stability was one of the most important factors. The stability of a plasmid may be affected by the genetic characteristics of host cells, the copy number of the plasmid, culture conditions, and the genes carried on the plasmid. Some proposals to ensure plasmid stability have also been reported (5). In this chapter, a new category is introduced. As a model system we employed the recombinant plasmid pSC101trpI15-14, which has a whole *trp* operon of *E. coli*. Since we isolated a mutant strain that maintained the plasmid very stably, the mechanism for the plasmid stability was investigated.

MATERIALS AND METHODS

Materials

Tetracycline (Tc), chlortetracycline, fusaric acid, and carbonylcyanide-m-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Corp., St. Louis, MO, USA. 6-Fluoro-DL-tryptophan was from Aldrich Chemical Co., Milwaukee, WI, USA. Tryptone and yeast extract were from Difco Laboratories, Detroit, MI, USA. [Side chain 2,3-^{3}H]-tryptophan was from New England Nuclear, Boston, MA, USA.

A DNA labeling and detection kit was from Boehringer Mannheim, Mannheim, FRG. Nylon membranes were from Pall Corp., Cortland, NY, USA. Gene-Clean was from Biol01 Inc., La Jolla, CA, USA. The RA Millipore filter was from Millipore Corp., Bedford, MA, USA. All other reagents were from Wako Chemical Industries, Osaka, Japan.

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The symbols *trpAE1, trpR,* and *tnaA* indicate a deletion mutant of the whole *trp* operon, a *trp* repressor-deficient mutant, and a tryptophanase-deficient mutant, respectively.

TABLE 1. Bacterial strains and plasmids

Strain/plasmid	Relevant properties/phenotype	Source/reference
E. coli W3110		
Tna	*trpAE1 trpR tnaA*	(3)
6F484	6-fluorotryptophan resistant mutant of Tna(pSC101trpI15-14)	This work
FA14	6F484 cured of pSC101trpI15-14	This work
Plasmid		
pSC101	Tc^r	(1)
pSC101trpI15-14	Tc^r Trp^+ (whole *trp* operon) I^-	(3)

Tc^r, Tetracycline resistance.
I^-, Insensitivity to feedback inhibition by tryptophan.

Media

L broth contained 10 g of Bacto tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of deionized water; it was adjusted to pH 7.5 with NaOH and was solidified with 15 g of agar per liter (L agar). Minimal medium was Vogel & Bonner's medium supplemented with 2 g of glucose per liter (1). For the selection of tetracycline sensitive (Tc^s) cells, the medium of Maloy et al. (6) was slightly modified as follows: agar 15g/*l*, Bacto tryptone 5 g/*l,* yeast extract 5 g/*l,* NaC1 10 g/*l*, NaH2PO4.H20 10 g/*l*, chlortetracycline. HC1 50 mg/*l,* fusaric acid 14 mg/*l,* tryptophan 2 mg/*l*, ZnCI: (20 mM solution) 5 ml, pH 7.5. NaH2PO4. H20, fusaric acid, tryptophan, and $ZnCl_2$ were separately filtered. Heat-inactivated chlortetracycline was used as an inducer of the Tc^r gene product.

Preparation of plasmid and chromosomal DNA

Plasmid DNA was prepared by the standard method (7). Chromosomal DNA of *E. coli* was prepared by phenol treatment procedure (8) and purified with Gene-Clean.

Transformation of *E. coli* with plasmid DNA

Transformation of *E. coli* was done by the method of Hanahan (9). Transformants with pSC101 or pSC101trpI15-14 were selected on L agar containing Tc (25 μg/ml) (L-Tc25 agar), and the tryptophan-producing ability was examined on minimal agar medium.

Mutagenesis

An exponentially growing culture of *E. coli* in L broth at 37°C was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (3).

Positive selection of Tc sensitive strain

Bacteria carrying Tc^r plasmid were cultured in L broth at 37°C overnight. Culture broth that contained $l0^7$ cells was plated on the selective agar for Tc^s cells (6, 10). After two days of incubation at 37°C, some large colonies which appeared among many tiny colonies as the lawn were transferred to L agar with toothpicks and examined for Tc^r/Tc^s on L-Tc25 agar.

Southern transfer

Chromosomal DNA and plasmid DNA were electrophoresed in agarose dissolved in Tris-acetate buffer (pH 8.0). Then DNA was denatured in the solution of 1.5 M NaC1 and 0.5 M NaOH, neutralized by 3 M sodium acetate buffer, and transferred to a nylon filter by electroblotting. The filter was baked and DNA was detected with a DNA labeling and

detection kit (11).

Stability test of the recombinant plasmid

As the first step, bacteria carrying the recombinant plasmid were precultured in L-Tc25 at 37°C overnight. Secondly, 50 μ1 of the culture broth was inoculated in 100 ml of fresh L broth and incubated at 37°C until late-exponential phase. This second process was repeated for about 100 generations of cell growth. Samples were taken and plated on L agar. Phenotypic stability of the cells was examined by replica plating on L-Tc25 agar and minimal agar as mentioned previously (1).

Active transport of tryptophan

Bacteria were grown to exponential phase (OD600≒1.0) in L broth at 37°C and incubated for another 30 min at 20°C. [2, 3-^{3}H]-Tryptophan (final concentration 33,000 cpm/ml) and cold carrier tryptophan (final concentration 20 μg/ml) were added to the culture broth. After incubation for various periods, 1 ml of culture broth was filtered on an RA millipore filter (1.2 μm pore size, 25 mm in diameter). These disks were washed three times with 10ml of L broth, kept at room temperature, dried, and counted in the Beckman liquid scintillation counter system LS-7500 (Beckman Instruments, Inc., Fullerton, CA, USA). To measure the non-specific adsorption of tryptophan to the cells, active transport of tryptophan was inhibited by the addition of uncoupler CCCP (12). CCCP was added to the exponential phase culture broth to a final concentration of 5 μM. To exhaust intracellular ATP, extra incubation at 37°C for 30 min was done and then the sample was used for the uptake experiment. The difference between the data with and without CCCP treatment corresponds to active transport of tryptophan. One optical density unit at 600 nm corresponded to 0.49 mg of dry cells per ml.

RESULTS

Isolation of a stable plasmid carrier mutant

E. coli Tna (pSC101trpI15-14) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the mutants that were resistant to 6-fluorotryptophan (1000 μg/ml) were selected. Eighty mutant strains were obtained, a frequency of around 10^{-9}. Plasmid stability was examined on the basis of Tc^r and Trp^+ for all the mutant strains. Among them, an extremely stable plasmid carrier was found. This strain, 6F484, stably maintained the recombinant plasmid even for more than 100 generations.

Curing of recombinant plasmid from strain 6F484

It has been reported that Tc^r bacteria produce TET proteins to prevent tetracycline permeation across the membrane (10). TET proteins bind to metal ions, and therefore Tc^r cells are hypersensitive to lipophilic chelating agents such as fusaric acid. Only Tc^s colonies can grow well on the agar plates containing fusaric acid. The simple technique allows direct plate selection of Tc^s clones from a predominant Tc^r population (10). Thus, the strain FA14 cured of pSC101trpI15-14 was obtained from strain 6F484, and the phenotype was confirmed to be Tc^s and a tryptophan auxotroph (Trp^-). A Southern transfer experiment proved that strain FA14 did not have any homologous segment with pSC101trpI15-14 (photograph not shown).

Stability of the recombinant plasmid

To examine the factor (host cell or plasmid), which may be responsible for the plasmid stability, *E. coli* strains Tna and FA14 were transformed with pSC101trpll5-14. The transformants were cultured and their phenotypic stability was investigated (Fig. 1). Strain Tna (pSC101trpI15-14) gradually lost the characteristics of Tc^r and Trp^+. After 99 generations, only 28% of the total bacteria had Tc^r, which allows the growth on the minimal medium. In contrast, the phenotype of strain FAl4 (pSC101trpI15-14) was stable. After 100 generations, all the population tested was Tc^r and Trp^+. When pSC101trpI15-14 which had been prepared from strain 6F484 was used to transform strain Tna, the transformant showed nearly the same plasmid stability as that of Tna (pSC101trpll5-14). It was therefore concluded that the extremely high plasmid stability was due to mutation in host strain FA14 and not to the recombinant plasmid.

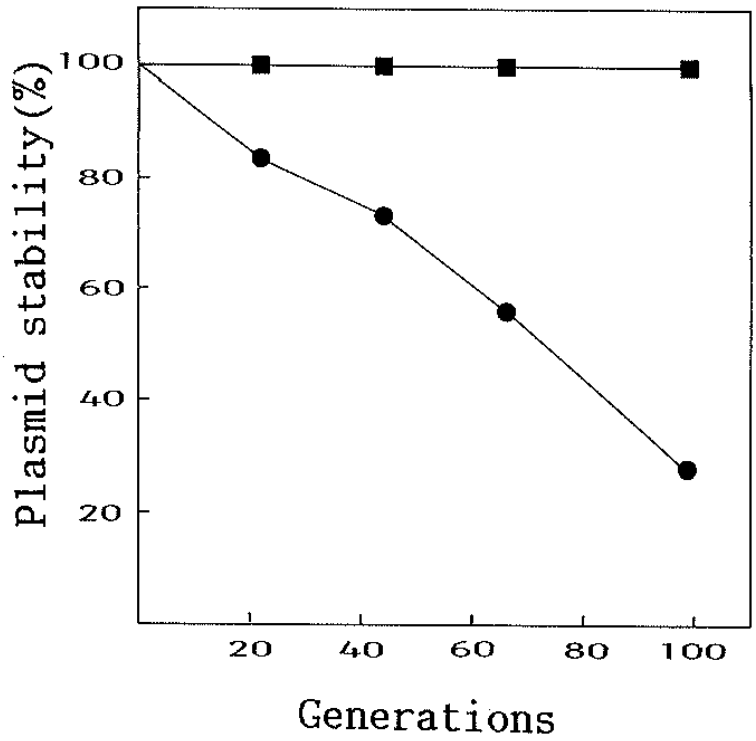

FIG. 1. Stability of plasmid pSC101trpI15-14 during cell growth. Phenotype of the plasmid is Tc^r, Trp+. Symbols: ●, Tna (pSC101trpI15-14); ■, FAl4 (pSC101trpI15-14).

Growth rate of *E. coli* cells with and without plasmid

I examined the growth characteristics in L broth at 37°C of the strains Tna, FA14, and their transformants with pSC101 or pSC101trpI15-14 (Fig. 2). FA14 grew more slowly than Tna; their specific growth rates were 0.50 h^{-1} and 1.31 h^{-1}, respectively. The growth rates of FA14 and FA14 (pSC101) were nearly the same. However, the growth of strain FA14 was stimulated by the transformation with pSC101trpIl5-14 and the specific growth rate of the transformant was very similar to those of Tna cells with and without pSC101trpIl5-14 (Fig. 2). These results indicate that the growth of strain FA14 was enhanced by the addition of the whole *trp* operon that most probably corresponds to the ability of tryptophan biosynthesis, even in a complete medium. It was therefore inferred that host strain FA14 could not efficiently take up tryptophan from the medium.

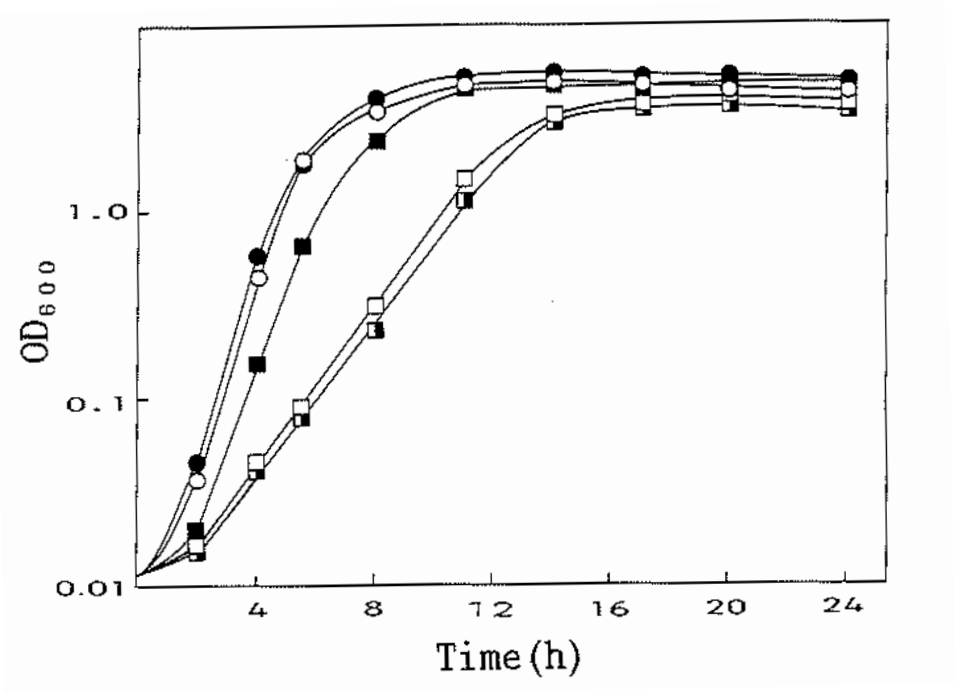

FIG. 2. Growth curves of *E. coli* cells with and without plasmid in a complete medium at 37°C.
Symbols: ○, Tna; ●, Tna (pSC101trpI15-14); □, FA14; ◨, FA14 (pSC101); ■, FA 14 (pSC101trpI 15-14).

Active transport of tryptophan

Tryptophan uptake by both strains, Tna and FA14, was examined using radioactive tryptophan. Since tryptophan uptake at 37°C was extremely fast to ensure the accurate measurement (data not shown), the experiment was done at 20°C (Fig. 3). Tryptophan uptake, including both active transport and adsorption to the cell surface, by strain Tna continued for 2 min and leveled off at a higher value than that of strain FA14. The level of ^{3}H-tryptophan for FA14 was constant after 15 seconds, and the incorporation of radioactive substance might be considered as the adsorption to the periplasm of cells. To eliminate the effects of active transport, an uncoupler, CCCP (inhibitor of ATP biosynthesis), was added to the reaction system. Consequently only the cell-bound tryptophan could be measured (Fig. 4). In fact, both strains Tna and FA14 showed constant values from 15 s to 6 min. The difference between the values shown in Fig. 3 and Fig. 4, probably due to the active transport of tryptophan. *E. coli* Tna had typical active transport of tryptophan, but strain FA14 showed little capacity of the active transport system. It is concluded from these results that strain FA14 lacks the active transport system for tryptophan, resulting in a low growth rate even in a complete medium. The slow growth of FA14 in a rich medium might be mainly supported by the nonspecific incorporation of tryptophan.

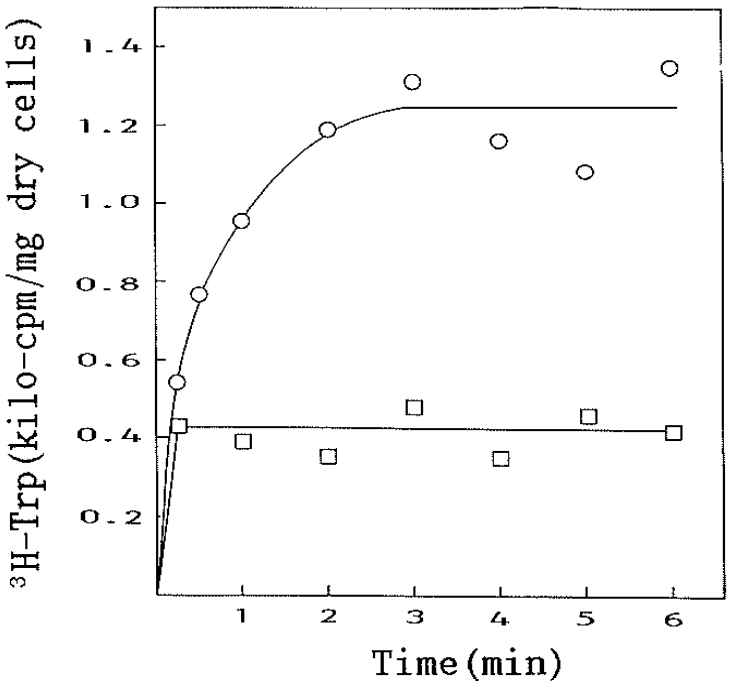

FIG. 3. Uptake of [^{3}H]-tryptophan by *E. coli* cells at 20°C. For details, see text. Symbols: ○, Tna; □, FA14.

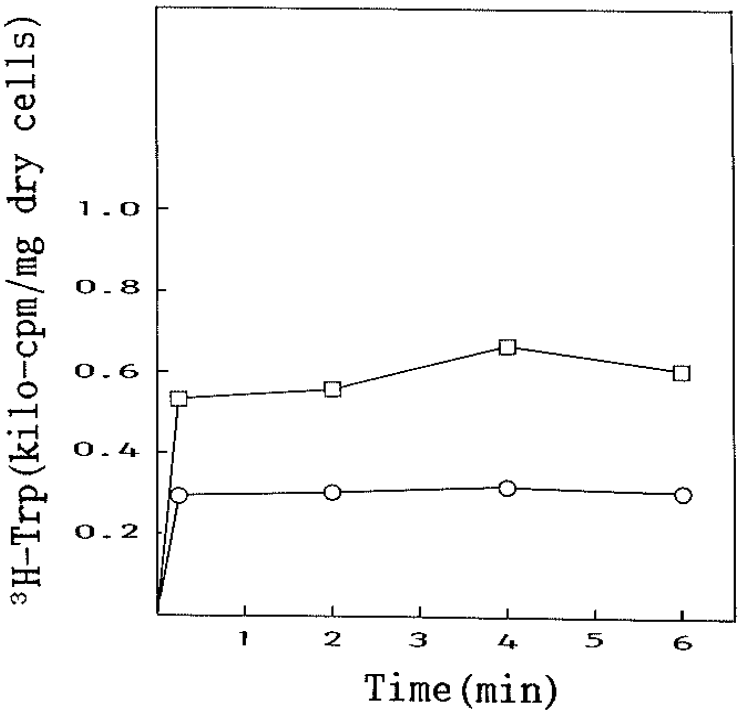

FIG. 4. Adsorption of [^{3}H]-tryptophan to *E. coli* cells at 20°C. CCCP (inhibitor of ATP biosynthesis), was added to the reaction system. Symbols: ○, Tna; □, FA14.

DISCUSSION

In this study, an extremely stable host-recombinant plasmid system was established. This system has the following characteristics: (i) deletion of the tryptophan operon from the host chromosome; (ii) a functionary defective active transport system of tryptophan in the host cell; and (iii) transformation of the host strain with a recombinant plasmid carrying the tryptophan operon (Fig. 5).

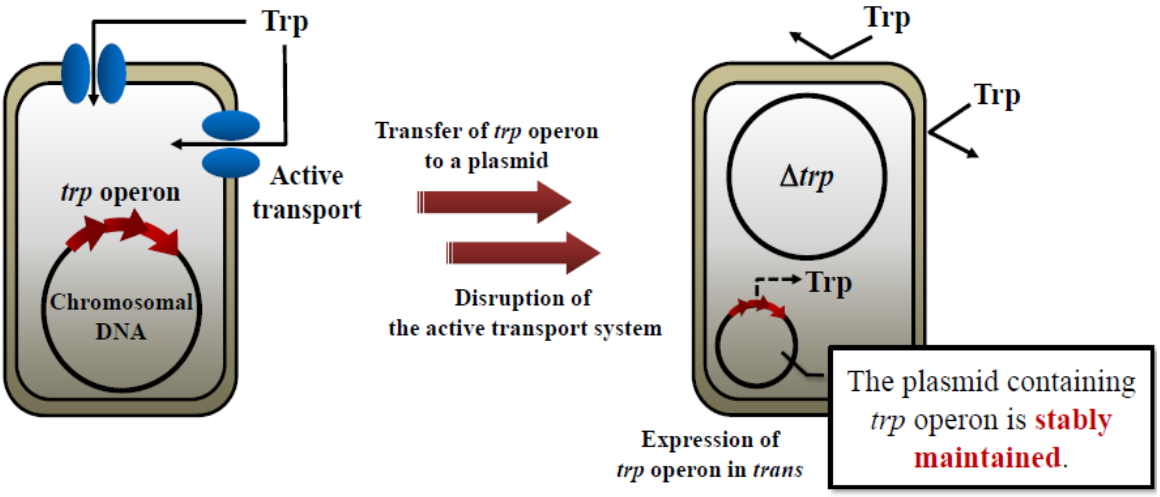

FIG. 5. Schematic diagram of the stable recombinant plasmid system based on tryptophan production.

The tryptophan auxotrophic mutant Tna of *E. coli* could not grow in the minimal medium, which does not include tryptophan. When it was transformed with pSC101trpI15-14, the transformant can grow normally even in the minimal medium, and the recombinant plasmid was fairly stable because cell growth depends on the plasmid. However, in a complete medium, the host cell was able to take up tryptophan from the medium and cell growth did not depend on the tryptophan operon in the plasmid. Therefore, the plasmid was not stably maintained. If the host strain is unable to take up tryptophan

efficiently, the *trp* operon recombinant plasmid carrier can preferentially grow even in a complete medium, which allows the plasmid to be stably maintained. Therefore, the three characteristics mentioned above are simultaneously required for the stability of the recombinant plasmid.

The aromatic amino acids are transported into *E. coli* K-12 by several transport systems (13). The common aromatic amino acid transport system transports all three aromatic amino acids, and three additional systems each transport a single aromatic amino acid, either tryptophan, phenylalanine, or tyrosine. In addition to these four systems, there is an inducible system for transporting tryptophan (14, 15). To date, at least three tryptophan transport systems have been found. When we isolated a mutant deficient in active transport of tryptophan from mutants resistant to 6-fluorotryptophan (1000 μg/ml), the frequency of mutation was very low, as mentioned in the Results. A possible explanation is that simultaneous mutations of multiple tryptophan transport systems are needed for a substantial deficiency of the substrate acquisition. Since the active transport-deficient mutant strain FA14 grows slowly in a complete medium, another transport system may be functional and/or diffusion of tryptophan may be occurring.

Another host-vector system for the selection and maintenance of plasmid carriers in antibiotic-free media has been reported (16). It is composed of a streptomycin-dependent (Sm^d) *E. coli* host and a plasmid vector carrying an *rpsL* gene, which masks the Sm^d phenotype. When this system is used for hyper-production of useful biomaterials, the specific gene must be cloned in the vector plasmid and the structural stability of the recombinant plasmid should be tested, because the heterologously introduced gene can easily be deleted during culture (5). By contrast, the new method developed in this study offers stable maintenance of the recombinant plasmid in the host cell during culture. Since the large-scale production of useful biomaterials generally involves a complete medium,

this concept is useful and might be applicable to production systems for any substances, such as amino acids and vitamins, which are essential materials for host cell growth.

Acetic acid bacteria are most commonly used for vinegar production. These bacteria produce energy effectively by allowing an electronic transmission transport system to link ethanol oxidation with the cell membrane-binding dehydrogenases, and accumulate acetic acid in the external environment. Acetic acid bacteria grow fairly well in the presence of ethanol and acetic acid, which are usually poisonous (17). *Gluconacetobacter europaeus* is the main acetic acid bacterium used in producing high-acidity vinegar in a bioreactor. It has a particularly high ethanol oxidation ability and acetic acid tolerance, showing tolerance to up to 20% acetic acid. Because of these characteristics, acetic acid bacteria can grow dominantly with no contamination if acetic acid is added to the culture medium. As a result, the fermentation process rule becomes simple; therefore, acetic acid bacteria are considered to be very advantageous in material production (17). Recently, cryptic plasmids have been found in *G. europaeus* KGMA0119 (18). These plasmids could have applications for the genetic manipulation of *G. europaeus*.

Comparative genomics suggests that *G. europaeus* possesses a biosynthetic pathway for the production of branched-chain amino acids (BCAAs) similar to those identified in the well-studied BCAA-producing bacteria *Corynebacterium glutamicum* and *Serratia marcescens* (Fig. 6). Alpha-amino butyric acid (ABA) is a well-known valine analogue that inhibits growth by inducing feedback inhibition during BCAA biosynthesis. *G. europaeus* M0119 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and ABA-resistant (ABAr) mutants were isolated. Almost all the ABAr mutants showed valine and leucine accumulation in the culture medium, whereas the wild-type strain did not. These results suggest that *G. europaeus* could be used as a BCAA-producing bacterium (19). If a mutant deficient in the BCAA transport system were isolated, *G. europaeus* would enable efficient

BCAA production using the extremely stable host-recombinant plasmid system that we have established in *E. coli*.

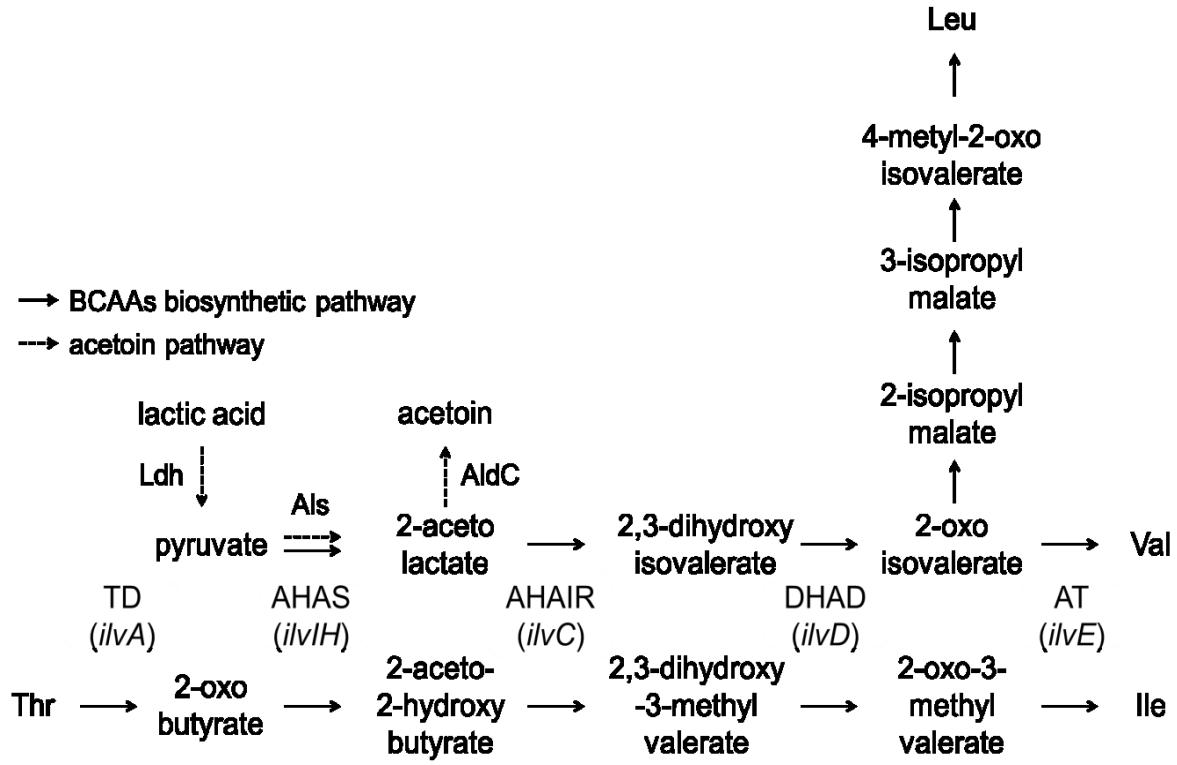

FIG. 6. Putative biosynthetic pathway for branched chain amino acids (BCAAs) in *G. europaeus* predicted from draft genome sequences of the type strain LMG18890^T (20). The lines with arrows and the dotted lines with arrows indicate the biosynthetic pathway for BCAAs and the acetoin pathway, respectively. TD, threonine deaminase; AHAS, acetohydroxyacid synthase; AHAIR, acetohydroxyacid isomeroreductase; DHAD, dihydroxyacid dehydratase; AT, aminotransferase; Ldh, lactate dehydrogenase; Als, α-acetolactate synthase; AldC, α-acetolactate decarboxylase.

SUMMARY

A new method to stabilize recombinant plasmids extremely well was exploited using *E. coli* Tna (*trpAE1 trpR tnaA*) and pSC101trpIl5-14 (tetracycline resistance, whole *trp* operon) as a model system. We mutagenized the Tna strain carrying pSC101trpI15-14 and isolated a mutant 6F484 that stably maintained the recombinant plasmid for 100 generations. From 6F484, plasmid-free cells (tetracycline sensitive) were screened for on selective agar plates containing fusaric acid. The host strain FA14 was found to have lost the ability for active transport of tryptophan, in addition to the phenotype of Trp^-. Therefore, strain FA14 could not grow normally even in a complete medium. However, when the strain was transformed with a recombinant plasmid carrying the *trp* operon, its growth rate was almost restored to the original level. These results suggest that the recombinant plasmid is indispensable for the normal growth of host cells like FA14. Even if plasmid-free segregants appear during the cultivation, they cannot grow so rapidly and are diluted as a minority in total population. Consequently, owing to the deficiency of both the biosynthesis and uptake of tryptophan in host cell, the *trp* operon recombinant plasmid can be stably maintained.

The acetic acid bacterium, *G. europaeus* has particularly high ethanol oxidation ability and acetic acid tolerance. And it can easily prevent various bacteria contamination by adding acetic acid to the medium. It is thought to be very advantageous for material production. Using cryptic plasmids found in *G. europaeus* KGMA0119, useful host-vector system is expected for further genetic modifications.

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GENERAL CONCLUSION

In this thesis, I described the cloning and sequencing of the thermostable alcohol dehydrogenase (ADH-T) gene (*adhT*) from *Geobacillus stearothermophilus* NCA1503 using *Bacillus subtilis* as a host and pTB524 as a vector plasmid. The deduced amino acid sequence (337 amino acid residues) was compared with the amino acid sequences of alcohol dehydrogenases (ADHs) from other organisms. The three-dimensional structure of the amino acid residues responsible for the catalytic activity of horse liver ADH has been determined. Since these catalytic amino acid residues are fairly well conserved in ADH-T and other ADHs, ADH-T is considered to have the same basic catalytic mechanism as horse liver ADH, which involves a zinc-bound water molecule, the hydroxyl group on Thr40, and the imidazole ring of His43. The putative catalytic system of ADH-T was studied by using site-directed mutagenesis to introduce point mutations at the catalytic amino acid residues Cys38 (a putative catalytic zinc ligand), Thr40, and His43. The following mutant enzymes were produced: Cys38Ser, Thr40Ala, Thr40Ser, and His43Ala. The cell lysates of the mutated strains of bacteria were used for ADH assays and SDS-PAGE. All mutant enzymes had the same level of expression. However, Cys38Ser, Thr40Ala, and His43Ala had no ADH activity. The activity of the mutant enzyme Thr40Ser was a little lower than that of the wild-type ADH-T, indicating that the hydroxyl group on serine can substitute for the one on threonine during catalysis. The Thr40Ser mutant had lower activity for primary alcohols than the wild-type enzyme; however, the mutant enzyme was more active for substrates with a larger side chain, such as 2-methyl-l-propanol and cyclohexanol. This phenomenon might be explained by the fact that the methyl group on threonine is not present on serine.

To change the *pK*a value of the putative system, His43 was replaced by the more basic amino acid, arginine. As a result, the optimum pH of the mutant enzyme His43Arg was shifted from 7.8 (wild-type enzyme) to 9.0. His43Arg exhibited a higher level of activity than the wild-type enzyme at the optimum pH. His43Arg exhibited a higher activity for primary alcohols (except 2-methyl-1-propanol) and acetaldehyde (as a reverse reaction) than the wild-type enzyme, but had little activity for secondary alcohols and ketones. The *K*m value of His43Arg for ethanol was fifty-fold larger than that of the wild-type enzyme.

The *aldhT* gene coding thermostable aldehyde dehydrogenase (ALDH-T) from *G. stearothermophilus* SIC1 was cloned by colony hybridization using *Escherichia coli* JM109 and pUC19 as a host-vector system. Nucleotide sequence analysis showed that the *aldhT* gene was composed of 1,464 bp (488 amino acid residues) that encode a protein with a molecular weight of 52,912. The *aldhT* gene was highly expressed in *E. coli* carrying the recombinant plasmid pUALD27R, which contains the native promoter for the *aldhT* gene. The enzyme accumulated in the soluble form, not in inclusion bodies in *E. coli,* and comprised approximately 10% of the total cytoplasmic protein. ALDH-T was purified to homogeneity with a specific activity of 36.3 U/μg protein by heat treatment of the cell extract, ammonium sulfate precipitation, gel filtration, and finally ion-exchange chromatography. The molecular weight of the native ALDH-T was estimated to be 220,000 by using gel filtration, whereas the subunit molecular weight, determined by SDS-PAGE, was 53,000, suggesting a tetrameric enzyme structure. ALDH-T was stable after heating at 65°C for 30 min, and the optimum temperature for enzyme action was 55–60°C. The enzyme was capable of oxidizing several aliphatic aldehydes, particularly C6-aliphatic aldehyde and hexanal, but did not oxidize benzaldehyde, an aromatic aldehyde. The deduced amino acid sequence of ALDH-T exhibited 43 % homogeneity to that of the human cytoplasmic aldehyde dehydrogenase. Two amino acid residues believed to be a part

of the active site, Cys289 and Glu255, were conserved among the several aldehyde dehydrogenases. Since the catalytic amino acid residues are fairly well conserved in several organisms including acetic acid bacteria, it was suggested that the cytoplasmic dehydrogenases (ADH and ALDH) of two acetic acid bacteria, *Acetobacter pasteurianus* and *Gluconacetobacter europaeus*, have the same catalytic mechanism.

In the final chapter, a material production system using a useful host-vector combination was discussed. A new, highly efficient method for stabilizing recombinant plasmids was developed using *E. coli* Tna (*trpAE1 trpR tnaA*) and pSC101trpIl5-14 (tetracycline resistance, whole *trp* operon) as a model system. We mutagenized the Tna strain carrying pSC101trpI15-14 and isolated a mutant 6F484 that stably maintained the recombinant plasmid for 100 generations. From 6F484, plasmid-free cells (tetracycline sensitive) were screened on selective agar plates containing fusaric acid. In addition to having the Trp^- phenotype, the host strain FA14 lost the ability for active transport of tryptophan and therefore could not grow normally even in a complete medium. However, when the strain was transformed with a recombinant plasmid carrying the *trp* operon, its growth rate was almost restored to the original level. These results suggest that the recombinant plasmid is indispensable for the normal growth of host cells like FA14. Even if plasmid-free segregants appear during cultivation, they cannot grow rapidly and are a minority in the total population. Consequently, owing to the deficiency of both the biosynthesis and uptake of tryptophan in the host strain, the *trp* operon recombinant plasmid can be stably maintained.

Acetic acid bacteria have a particularly high ethanol oxidation ability and acetic acid tolerance. Preventing contamination of the culture by other bacteria is easily done by adding acetic acid to the medium. This characteristic of acetic acid bacteria makes it very useful as a host cell for large-scale material production. Cryptic plasmids were found in *G.*

europaeus KGMA0119, which could contribute to the development of a useful host-vector system using acetic acid bacteria as we developed in this study.

LIST OF PUBLICATIONS

1. Cloning and Sequencing of the Gene Coding for Alcohol Dehydrogenase of *Bacillus stearothermophilus* and Rational Shift of the Optimum pH.
   Hisao Sakoda, Tadayuki Imanaka.
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2. Effect of Amino Acid Substitutions at the Active Center on the Activity of *Bacillus stearothermophilus* Alcohol Dehydrogenase.
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3. Cloning, Nucleotide Sequence, and Efficient Expression of the Gene Coding for Thermostable Aldehyde Dehydrogenase from *Bacillus stearothermophilus*, and Characterization of the Enzyme.
   Tadayuki Imanaka, Toyoo Ohta, **Hisao Sakoda**, Nunuk Widhyastuti, Masayoshi Matsuoka.
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4. Cloning and Nucleotide Sequence of the Gene Coding for a Subunit of the Respiratory NADH Dehydrogenase from *Bacillus stearothermophilus.*
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5. A New Way of Stabilizing Recombinant Plasmids.
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6. New Utilization of Acetic Acid Bacteria.
   Hisao Sakoda, Naoki Akasaka, Shinsuke Fujiwara.
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